Trigonella foenumgraecum L. aseptic cell cultures and their steroids.

Stevens, Robert George

Award date:
1974

Awarding institution:
University of Bath

TRIGONELLA FOENUMGRAECUM, L.

ASEPTIC CELL CULTURES AND THEIR STEROIDS

submitted by

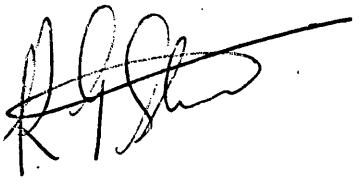
ROBERT GEORGE STEVENS

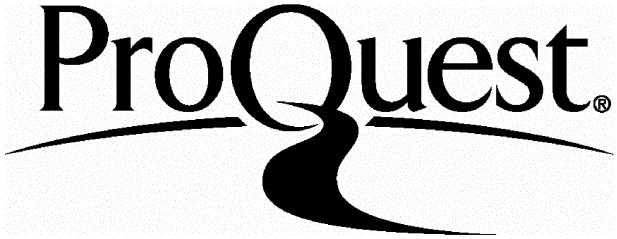
for the degree of

Doctor of Philosophy

of the University of Bath

1974





Summary

A description of aseptic plant cell culture, including a glossary of terms, has been given and the biosynthesis of phytosterols and steroidal sapogenins reviewed.

The method and conditions employed in the induction of callus cultures from the cotyledons of Fenugreek seed are described. The growth of callus cultures has been studied and expressed as the increase in fresh weight, when using partly and fully defined media with different growth regulator regimes.

Phytosterols and steroidal sapogenins were detected in cultures maintained for one year on the induction medium. These compounds were isolated and identified using gas liquid chromatography, infared analysis and mass spectrometry. Unsuccessfull attempts were made to determine the small quantities of diosgenin and yamogenin present in the callus cultures by a colourimetric assay originally designed for seed samples. A successful assay method was devised which involved separation of the phytosterol and sapogenin fractions by adsorption column chromatography and subsequent assay of both fractions by gas liquid chromatography. The conditions affecting the yield of the aglycone sapogenin from the callus tissue have been examined.

Considerable differences were observed in the composition of the free sterol and sapogenin components from callus cultures grown either with NAA or 2,4-D. No significant changes in the steroid yield of cultures was caused by altering the vitamin formulation of the medium, introducing a rare amino acid isolated only from Fenugreek seed, or varying the light conditions under which the cultures were

grown.

Experiments were performed to select a defined medium for the initiation and maintenance of Fenugreek suspension cultures. The basal medium used for callus culture proved satisfactory, but a reduction in the concentrations of the growth regulators was necessary to induce a cell suspension. The sapogenin yield of the suspension cultures was lower than that of the callus culture from which the original explants were taken.

ACKNOWLEDGMENTS

I am indebted to Professor D. A. Norton, Head of the School of Pharmacy, for providing facilities for this research, and especially my Supervisor, Dr. Roland Hardman, for his encouragement and advice during these studies.

I should also like to thank Dr. T. M. Jefferies and Dr. N. Sunderland for their technical assistance and Mr. R. Sadler for his help in the preparation of the figures in this thesis. I am grateful to the Science Research Council for the award of a studentship which enabled me to undertake this work.

My gratitude is also due to my wife and parents for their constant understanding and support.

CONTENTS

PART II

PART III EXPERIMENTAL

PART III

PART III

LIST OF FIGURES

PART I INTRODUCTION

PART II RESULTS AND DISCUSSION

LIST OF TABLES

PART II RESULTS AND DISCUSSION

PART III. EXPERIMENTAL

PART I - INTRODUCTION

CHAPTER I

ASEPTIC PLANT CELL CULTURE

INTRODUCTION

Aseptic plant cell culture is the growing of plant material under aseptic conditions on a nutrient medium. The term covers techniques for the culture of organised plant material, such as sterile seedlings and isolated plant organs, the culture of undifferentiated cell masses initiated from segments of plant tissue and the culture of cell masses derived from single plant cells.

Street[1] has attempted to rationalise the terminology used in plant cell culture, which previously lacked uniformity. A list of the relevant terms which he has defined has been included.

(a) Plant culture - the culture of seedlings or larger plants.

(b) Organ culture - the culture of isolated organs including root tips, stem tips, leaf primordia, immature parts of flowers and immature fruits.

(c) Tissue or callus culture - tissues arising by proliferation from segments (explants) of plant organs.

(d) Suspension cultures - isolated cells or small aggregates remaining dispersed as they grow in liquid medium.

(e) Clone - tissue from a single explant, which is maintained by repeated subculture of a many-celled piece of the parent culture.

(f) Passage - a single incubation period between subcultures.

(g) Established or permanent cultures - cultures which have been maintained through many successive passages.

(h) Inoculum - a fragment of the parent culture, subcultured by transfer to new medium (sometimes referred to as an explant).

(i) Organogenesis - the development of shoot buds or roots from tissue cultures or suspension cultures.

Haberlandt[2] first attempted, but failed, to grow isolated plant cells on a nutrient medium in 1902. In 1934 White [I] reported the establishment of an actively growing clone induced from tomato roots. In the same year Gautheret [I] established cultures (cambium cells of Salix capraea) which proliferated and grew 'algae-like' growths on a solid medium containing mineral salts (Knop's solution) glucose, and the amino acid derivative cysteine hydrochloride. White's [3] discovery of the importance of the B vitamins and the recognition of the importance of the growth regulator, auxin, isolated and identified as indole-3-acetic acid (IAA) led Gautheret [4] to include both in his media. In 1939 [5] he succeeded in growing cultures, from explants of carrot tissue, which satisfied the two major criteria of tissue culture, potentially unlimited growth and undifferentiated growth.

Subsequent work on tissue culture, by many workers, resulted in cultures which were slow growing and tended towards differentiation with age. In 1952 Steward[6] included coconut water in media for carrot cultures and obtained vigorous, undifferentiated growth with rapid cell division. This led to the wide use of coconut water in media and unsuccessful attempts were made to isolate the compound, now called a cytokinin, responsible for the growth and cell division. Work by Miller et al[7] (1955) resulted in the first compound exhibiting cytokinin activity being isolated from the DNA fraction of herring sperm. The use in culture media of this compound, kinetin, with the auxin, IAA, rapidly led to the development of various techniques of plant cell culture and allowed the establishment of cultures from many plant species. Muir established suspension cultures in 1953 (from callus of Tagetes erecta) and succeeded in growing cultures derived from single cells.[8] Recently cultures have been established from haploid cells[9] and plant cell protoplasts have been prepared.[10]

TISSUE CULTURE MEDIA

The unorganised tissue cultures derived from different plant species require similar macronutrients, micronutrients and an organic carbon source. The individual requirements of tissue cultures from different species for an organic source of reduced nitrogen, increased vitamin supplement, growth regulators and complex supplements (coconut water and yeast extract) varies. Many media have been designed to fill the specific requirements of particular tissues and some,

particularly White's medium (1943) and Murashige and Skoog's revised tobacco medium (1962) have been used, with minor modifications, for cultures from a great number of species.

GROWTH REGULATORS

Plant growth regulators are organic chemicals which stimulate, inhibit, or in some way alter growth and development. Growth and differentiation are controlled by the interaction of several different types of regulator, which have been conveniently classified into five distinct groups;[11] auxins, gibberellins, cytokinins, abscisic acid and ethylene. The requirements of tissue cultures for growth regulators varies with species.

Tissue cultures of Trigonella foenumgraecum (Fenugreek) grown in the experiments described in this thesis required both auxin and cytokinin for sustained healthy growth. The effects and possible mechanisms of action of auxin and cytokinin have, therefore, been considered.

(1) Auxins

Auxins are substances which promote cell enlargement, inhibit root growth, stimulate the production of adventitious roots and inhibit leaf and fruit fall. The plant auxin, identified as indole-3-acetic acid (IAA), was isolated from yeast and fungi in 1935 and subsequently from higher plants in 1942-1946.[12] It is synthesised from tryptophan in plant cells and metabolised in three ways.

(i) By the mechanisms involved in growth

(ii) By enzymatic oxidation

(iii) By detoxification through conjugation with other components

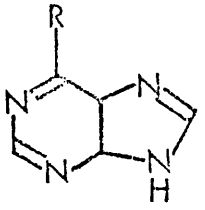
The mechanisms by which the natural auxin (IAA) and synthetic auxins (naphthalene acetic acid and 2,4-dichlorophenoxy acetic acid) induce cell elongation has been extensively studied. The auxins increase the plasticity of the cell walls, which are then stretched by water uptake resulting from the osmotic potential of the vacuolar sap.[11] The wall deformation is thought to be brought about by the breaking of crosslinks between the cellulose microfibrils of the wall. Cell wall extension is irreversible and subsequent resynthesis of the crosslinks of the elongated wall occurs. Auxins promote cell wall synthesis, but only after initial wall extension. Key[13] obtained an increase in cellular protein and RNA during auxin induced cell wall extension. Masuda and Kamisaka[14] studied RNA synthesis during IAA induced cell wall extension in oat coleaptiles. Cell extension occurred 12-13 minutes after the addition of IAA, whereas RNA synthesis was detected after 10 minutes. They postulated that IAA directly increased the template activity of DNA, increased the synthesis of m-RNA and the subsequent synthesis of protein. Increased protein synthesis was thought to result in the synthesis of a cell wall degrading enzyme necessary for breaking the cell wall crosslinks. Increased amino acid incorporation was observed as a result of IAA treatment in pea internodes[15] and IAA induced cell extension has been inhibited by protein synthesis inhibitors, such as chloramphenicol. Merkys et al[16] have shown a correlation between the binding of IAA with DNA and RNA conjugates and cell elongation and concluded that the

primary step of IAA growth acceleration was the interaction of IAA with DNA and RNA and its effect on protein synthesis. Galston and Davies[II] suggested that while such a mechanism probably explained the continued action of auxin, initially a different mechanism was involved. While auxin induced growth has been observed in 10 to 15 minutes in oat tissue, the time required for increased protein synthesis was four times longer. They suggested that either the initial action of auxin was to cause qualitative rather than quantitative changes in protein synthesis, or that auxin acted on some pre-formed system.

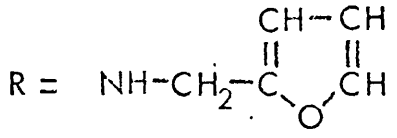
Substances such as naphthalene acetic acid (NAA) and 2,4-dichlorophenoxyacetic acid are not found naturally in plant tissues, but at suitable concentrations exhibit properties similar to IAA. The specificity of IAA metabolising enzymes means that these compounds are more persistent in their action and they have been widely used in tissue culture in place of IAA.

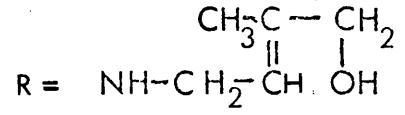
(2) Cytokinins

The other fundamental process of plant growth is cell division and naturally occurring compounds which can influence this process are known as cytokinins. Cytokinin activity was observed as a result of adding coconut water to culture media. Caplin and Steward[6] used coconut water with carrot root cultures and obtained vigorous growth and cell division. As already mentioned, in 1955 Miller et al[7] isolated kinetin (6-furfurylamino purine) which has since been widely used in tissue culture media. In 1964 Letham[I] isolated a naturally

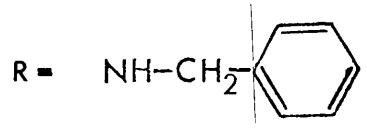


Kinetin





Zeatin



Benzylaminopurine

occurring plant cytokinin from sweet corn which he named zeatin. Like kinetin, and most of the other compounds since found with cytokinin activity, it was a 6-substituted adenine derivative.

Apart from influencing cell division, cytokinins have been observed to have several other properties. Kinetin can cause shoot initiation, bud formation, root growth, leaf growth and flowering and inhibit senescence in plants.[17] Like auxins, the mechanisms of cytokinin action appears to be closely involved with the regulation of nucleic acid or protein synthesis. Recent work suggests that cytokinins have been detected by bioassay in hydrolysates of t-RNA.[17] from both higher plants and animals. In serine t-RNA the substance N^6-(Δ^2-isopentenyl adenine) (IPA), which exhibits cytokinin activity, was an integral part of the structure

adjacent to the anticodon and was found to be necessary for attachment of the t-RNA to the m-RNA. A labelled cytokinin (C^{14} labelled benzylaminopurine) was added to callus cultures dependent on exogenous cytokinin for growth. Although most of the added cytokinin was degraded, 1% was incorporated into t-RNA. Fractionation of the t-RNA showed that the labelled compound was present in only one sub-fraction, indicating incorporation into only certain t-RNA's. Other evidence contradicts these results. Attempts to show the incorporation of a different cytokinin (6-benzylamino-9-methylpurine) into t-RNA were unsuccessful, although the substance still exhibited cytokinin effects on the tissue growth. It has been suggested that cytokinin is not incorporated into t-RNA, but that the presence of cytokinins in t-RNA hydrolysates represents breakdown products rather than precursors.[11] The presence of IPA in t-RNA of serine may result from the attachment of isopentenyl groups, derived from mevalonic acid or Δ^3-isopentenyl pyrophosphate, to a specific adenine in preformed t-RNA. Subsequent hydrolysis of the t-RNA might then release the complete IPA molecule. It is possible that, although cytokinin molecules can be extracted from t-RNA, the site of action of cytokinin is elsewhere. Experiments with moss protonema showed that cytokinin caused bud formation. The continued presence of cytokinin was required throughout bud development, or the buds reverted to protonemal filaments. It was found that cytokinin was only loosely bound to 'target cells' and could be washed out.[13] In this system Galston and Davies[11] suggested cytokinin was acting by binding to a specific site in a responding cell. The mode of action of

cytokinins is, therefore, not clear at the present time and further investigations are required.

THE APPLICATIONS OF TISSUE CULTURE

The development, over the last ten years, of reliable techniques and equipment for the culture and aseptic manipulation of tissues has increased the research into possible applications for tissue culture. The control of nutritional and environmental conditions afforded by plant tissue culture have already made it an invaluable technique in plant physiology and biochemistry. The possibility of using cell cultures for the biosynthesis of economically important plant constituents was recognised in the early 1950's, but at that time techniques were too exacting and uncertain for general use. The development of large scale fermentors,[19] operational under aseptic conditions, has made the routine cultivation of large quantities of plant cells possible and product biosynthesis, with reference to commercial manufacture, has become relevant. Klein[20] has listed several advantages of plant product production by this technique.

(i) The precise control of the growth environment

(ii) The possibility of establishing physiologically and genetically uniform suspension cultures

(iii) The elimination of problems encountered in the processing of plant material, such as variation in uniformity and quality of raw materials, availability and damage in transport or storage

(iv) The ability to force synthesis in a given direction by the appropriate control of growth conditions and precursors

Naturally the range of compounds detected in tissue cultures is large and includes alkaloids, amino acids, antibiotics, carbohydrates, enzymes, flavonoids, glycosides, growth regulators, organic acids, phenols, pigments, proteins, saponins, steroids, tannins and terpenoids[21]. To be of real commercial value products chosen for biosynthesis by large scale cell culture will have to be those which are difficult to obtain from other sources and for which an increasing demand exists. Several types of pharmaceutically active substances satisfy these criteria, namely alkaloids, cardiac glycosides and steroids.

PLANT PRODUCT BIOSYNTHESIS IN TISSUE CULTURES

The biochemical potential of tissue and suspension cultures would be expected to be genetically the same as the plant tissue from which the cultures were induced.[22] However, it has proved difficult to induce cultured cells to form characteristic metabolites and storage products, which develop normally in the intact plant organ of origin. Metabolic activities normally appear to be mainly directed to the synthesis of metabolites required for growth and cell division. In work so far reported culturessynthesisingsolanaceous alkaloids have, at best, produced 10% of the amount present in the parent plant.[22] In studies of the ability of Digitalis lanata to produce cardiac glycosides yields of 0.002-0.02%m.f.b. compared with 0.1-0.2% m.f.b. in the intact plant have been reported[23].

The ability of cultures to synthesise characteristic products has been shown to be dependent on both the medium used

and on the origin of the culture clone. Conflicting reports on the ability of tobacco pith callus to produce nicotine exist.[22] Furuya reported the presence of 0.005% nicotine in his cultures, whilst Dawson reported that tobacco callus cultures lost the ability to synthesise nicotine after 28 days and Turner[22] was unable to detect alkaloid in his tobacco culture. Buchner and Staba[23] reported the presence of 12 compounds in Digitalis cultures responding to a locating agent on a thin layer chromatograph in a similar way to cardenolides. Turner[22] was unable to detect similar compounds in his Digitalis cultures.

The two major problems in the use of tissue culture for product biosynthesis are, therefore, the initiation of culture capable of biosynthesising the desired substance and stimulation of the culture to produce it in economical quantities.

THE USE OF TISSUE CULTURES FOR THE BIOSYNTHESIS OF STEROIDS

Since the first use of cortisone as a medicine in 1950, the number of pharmaceutically important steroids has expanded. The range now includes not only corticosteroids but fertility control compounds, sex hormones, anabolic agents and more recently a general anaesthetic (Althesin). Partial synthesis of these compounds from steroids obtained from plant and animal sources has proved to be cheaper than total synthesis and plant materials are more economical, and present fewer storage problems than animal tissues, as a source of raw material. In 1968 the world steroid consumption was equivalent to 1000 tons of diosgenin of which it was estimated,

5% was produced by total synthesis, 6% by partial synthesis from animal sources and 89% from plant sources. It was predicted that by 1973 world consumption would be equivalent to 1340 tons of diosgenin.[24]

Diosgenin, or a similar compound (e.g. stigmasterol, hecogenin or smilagenin) is normally extracted from the plant material and converted to the final pharmaceutically active steroid, or a close intermediate, in the country in which the plant grows. The export of plant material or the basic steroids is restricted.

The main plant sources of diosgenin are the members of the Dioscorea species, family Dioscoreaceae. The sapogenin is extracted from the perennial tubers of the plants which slowly increase in size and may survive for up to 20 years. Because of the slow growth of the tuber, organised cultivation has not proved economical and the plants are normally collected from their natural habitats, the lower slopes of the Himalayas and the tropical forests of Mexico and Central America. Although this procedure has so far managed to satisfy world demands for steroids it is evident that, with increased demand, an alternative source of supply will soon be necessary. Alternative plant sources of diosgenin have been studied, including the seed of Trigonella foenumgraecum, L. also known as Fenugreek.[24] Investigations are being carried out to develop a suitable strain for cultivation of this plant in a temperate climate but, even if successful, crop yield will still be dependent on uncontrollable environmental conditions.

Tissue cultures as a source of a suitable steroid for

partial synthesis could alleviate the dependence of the pharmaceutical industry on foreign producers or climatic conditions.

The first attempts at obtaining diosgenin biosynthesis from tissue cultures were, naturally enough, carried out with the species of Dioscorea. Cultures of Dioscorea deltoidea were established[25] and diosgenin was synthesised by both suspension and tissue cultures (1% m.f.b.). Subsequently cultures of Dioscorea composita, D. floribunda and D. speculiflora[26] were established and diosgenin detected in the tissue obtained.

As with cultures of other species, the ability of cultures initiated from Dioscorea species to produce diosgenin was not as great as that of the parent plants and in the case of Dioscorea composita some workers were unable to detect any diosgenin in their cultures[27 28], whilst in similar cultures grown by Mehta and Staba[26] diosgenin was detected (.06% m.f.b.).

FENUGREEK TISSUE CULTURES AS A POSSIBLE SOURCE OF STEROIDS

As has been previously mentioned, Trigonella foenumgraecum has been considered as an alternative plant source for the steroids needed for the partial synthesis of pharmaceutically active steroids. The steroid constituents of this species have been extensively studied[29 30 31 32] and the major sapogenin fraction has been shown to be a mixture of diosgenin and its 25S-epimer yamogenin (in a ratio of 3:2), occurring as their glycosides, both of which are acceptable in the partial

synthesis of steroids. The sapogenin level was found to be highest in the ripe seed (1-1.5% m.f.b.). The true phytosterols, cholesterol, campesterol and sitosterol (4-desmethyl sterols) have also been extracted from all parts of the plant in free, ester and glycoside forms.[29]

No published information was available on the aseptic plant cell culture of Fenugreek in 1971. The experiments in this thesis describe the preliminary investigations carried out into the possibility of establishing Fenugreek tissue and suspension cultures and their use for the biosynthesis of sapogenin or phytosterol.

In Part II, Chapter I of this thesis the selection and preparation of a medium suitable for the induction of Fenugreek callus cultures is reported. The techniques used in the induction of cultures from the cotyledons of sterile seedlings are also described. Chapter II reports the experiments performed to determine the combinations of cytokinin (provided as kinetin or as the complex supplement coconut water) and auxin [Naphthalene acetic acid (NAA) and 2,4-dichlorophenoxyacetic acid (2,4-D)] which give the optimum growth (increase in fresh weight) of the cultures. Both sapogenin (diosgenin/yamogenin mixture) and phytosterols (cholesterol, campesterol, stigmasterol and sitosterol) were biosynthesised by the callus cultures and Chapter III gives the techniques used to isolate and identify these compounds from one year old cultures. Chapter IV describes the unsuccessfull attempt to modify a colourimetric method for the assay of diosgenin and yamogenin extracted from young callus cultures. A G.L.C. method finally

adopted for the assay of sapogenin and free sterol is described in chapter V. The use of two different auxins, naphthalene - acetic acid and 2,4 dichlorophenoxyacetic acid resulted in considerable differences in the free sterol and sapogenin yield from callus cultures and these are reported in chapter VI. Attempts were also made to alter the sapogenin and sterol yield of callus cultures by alteration of the light conditions and by adding vitamins or, the amino acid 4-hydroxyisoleucine to the medium. These experiments are reported in chapter VII. Chapter VIII describes the experiments performed to determine suitable conditions for the growth of suspension cultures initiated from callus inocula.

PART I - CHAPTER II

THE BIOSYNTHESIS OF PHYTOSTEROLS AND STEROIDAL SAPOGENINS

The presence of 4,4'-desmethyl phytosterols and the sapogenins, diosgenin and yamogenin, in tissue cultures of Fenugreek was confirmed in the course of the work described in this thesis, (Part II, Chapter III). The biosynthetic pathway of these compounds is, therefore, outlined in this chapter.

THE BIOSYNTHESIS OF STEROLS

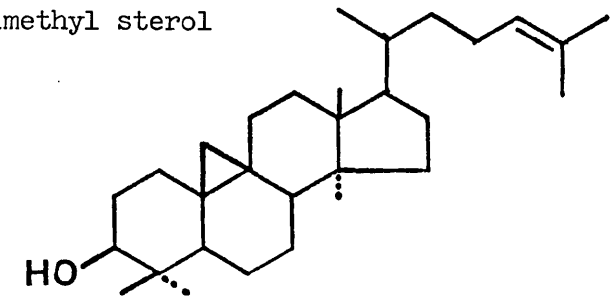
The biosynthesis of the common 3β monohydroxy sterols has recently been reviewed by Goad and Goodwin.[33] Sterols can be divided into three groups on both structural and biosynthetic growths, (a) the 4,4'-dimethyl sterols or triterpenes; (b) the 4-methyl sterols and (c) the 4-desmethyl sterols, referred to by Evans[34] as 'the true phytosterols'. Examples of each group are given in FigI.I . The common 4-desmethyl sterols normally found in angiosperms are stigmasterol, cholesterol, campesterol and sitosterol, the latter normally being the major component. These sterols have $\Delta 5$-unsaturation and a 24R-configuration at C-24.

SQUALENE BIOSYNTHESIS

The initial stages of sterol biosynthesis involve the formation of the C_{30} hydrocarbon squalene, from acetate and mevalonic acid, via a sequence of phosphorylated compounds. Cornforth et al[35] demonstrated with stereospecifically labelled ^{3}H- and ^{14}C-mevalonic acid that the R- form of mevalonic acid is incorporated into terpenoids and the S-form is metabolically inactive. The biosynthetic route to squalene was first demonstrated in animals[36] but the same route has been shown in plants[37 38 39 40] and is shown in Fig. I.2

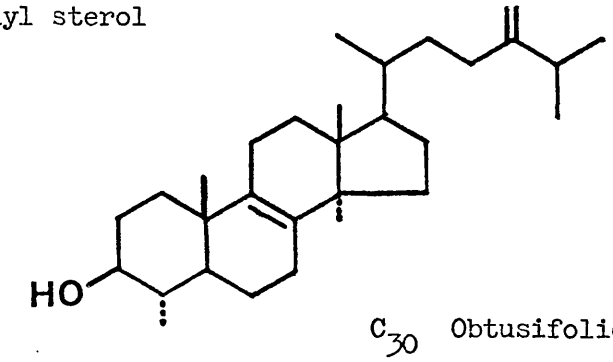
Fig I.I Some plant sterols

4,4'-dimethyl sterol



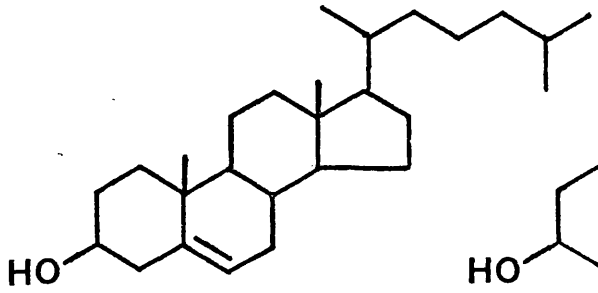
C_{30} Cycloartenol

4α-methyl sterol

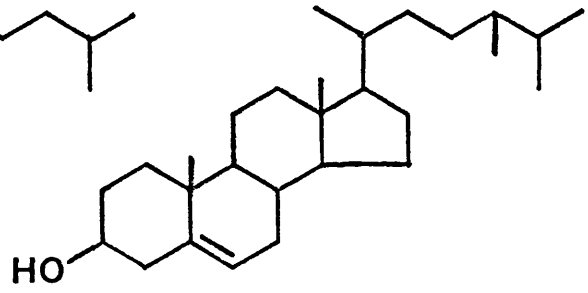


C_{30} Obtusifoliol

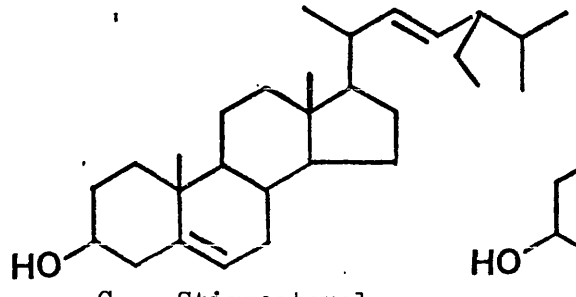
4-dimethyl sterols



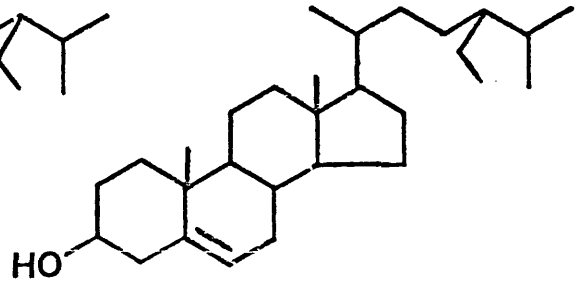
C_{27} Cholesterol



C_{28} Campesterol

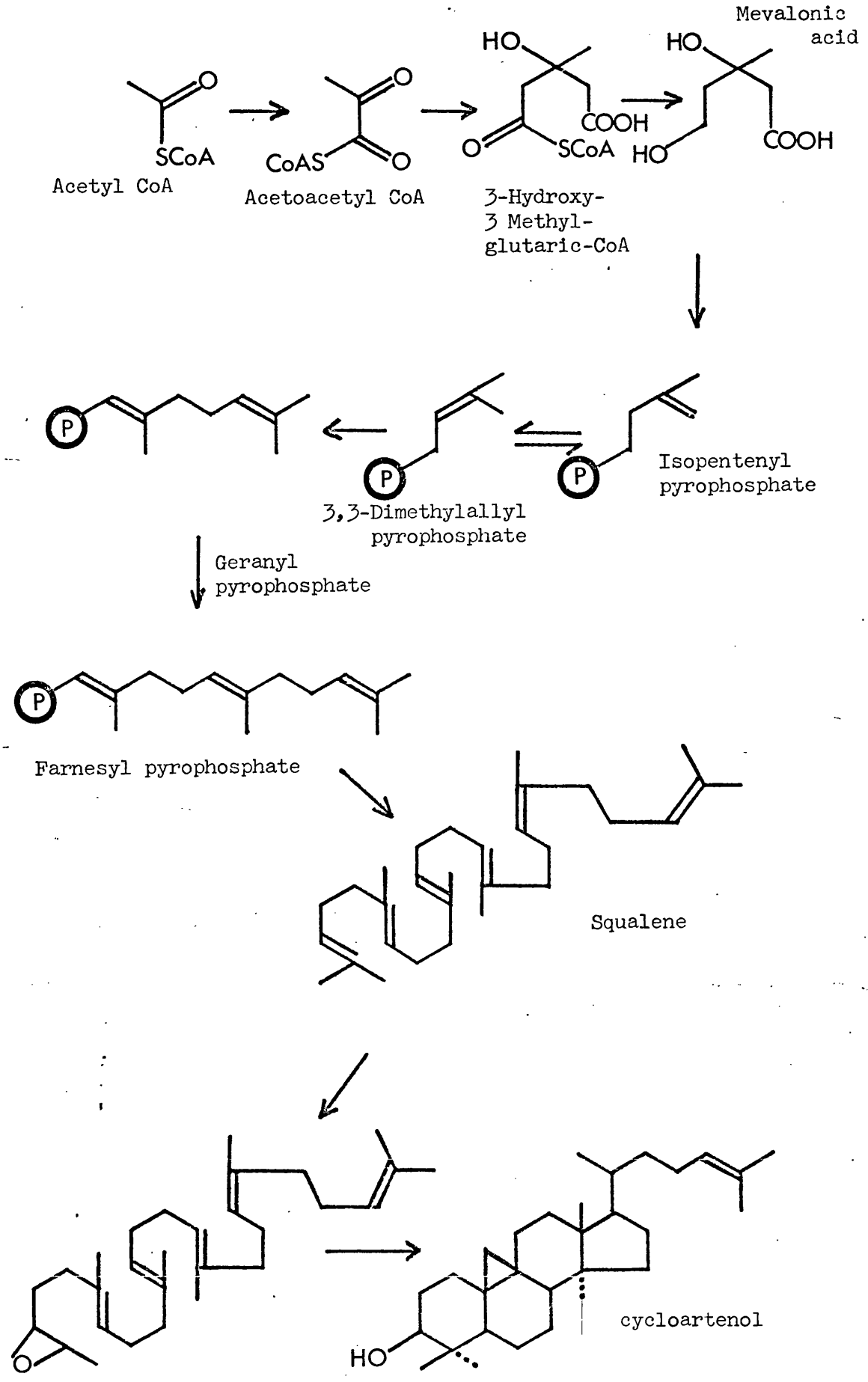


C_{29} Stigmasterol



C_{29} Sitosterol

Fig.I.2 Squalene biosynthesis and cyclisation



SQUALENE CYCLISATION

The mechanism of squalene cyclisation has been demonstrated in rat liver to proceed, in the presence of oxygen and NADPH, to the compound squalene-2,3-oxide.[41] This undergoes proton initiated cyclisation to yield lanosterol. In algae and higher plants cyclisation of squalene-2,3-oxide yields cycloartenol as the first identifiable product. Benveniste et al[42 43] obtained incorporation of 1-^{14}C-acetate into squalene-2,3-oxide and cycloartenol in Nicotiana tabacum tissue cultures and this was later confirmed with tissue cultures of Agave and Dioscorea[44] and using labelled mevalonic acid with Solanum tuberosum leaves.[45] No lanosterol was detected in any of this work and it was concluded that cycloartenol was the direct product of squalene cyclisation in these plants,(Fig. I.2).

Only one C-24 alkylation mechanism for the conversion of cycloartenol to the 4-desmethyl sterols has, so far, been considered in detail[33], Fig I.3 The alkylation of cycloartenol at C24 to give 24-methylene cycloartenol was proposed as the first step and this compound has been found to be widely distributed in plants.[46] Demethylation at C-4 to give rise to another widely reported plant compound cycloeucalenol[46] and opening of the 9B-19-cyclopropane ring to give the 4αmethyl sterol obtusifoliol would then occur. Obtusifoliol was first isolated from Euphorbia obtusifolia and has since been found in conjunction with cycloeucalenol in other plants.[34] Demethylation of obtusifoliol at C-14 and rearrangement of the nuclear double bond to $^{7}\Delta$ would yield methylene lophenol. The various C_{28} sterols (eg campesterol) would then be formed by C4deme thylation, to give 7-ene-24 methylene cholestenol, and $^{7}\Delta$ to $^{5}\Delta$ nuclear

double bond rearrangement, to give 24-methylene cholesterol; both compounds have been detected in Digitalis purpurea.[34]

A possible route to the C_{29} sterols involving a second alkylation of 24-methylene lophenol at C28, to give 24-ethylidene lophenol has been proposed and the latter is widely distributed in higher plants,[46] Fig.1.4 .The subsequent removal of the 4α-methyl group would give stigmasta-7,24(28)-dien-3β-ol[47] which could progress to 28-isofucosterol via a $\Delta^{5,7}$ intermediate stigmasta-5,7Z-24(28)-trien-3β-ol. Finally, reduction of the 24-ethylidene group of 28-isofucosterol would produce sitosterol. Reduction of the 24-ethylidene group at an earlier stage so that 24-ethylidene lophenol becomes stigmast-7-en-3β-ol has also been proposed.[33] Sitosterol would then be produced from this compound via the $\Delta^{5,7}$ intermediate stigmasta-5,7 dien-3β-ol.[48] Stigmasterol would be produced from stigmasta-7-en-3β-ol via spinasterol[33] and a $\Delta^{5,7}$ intermediate stigmasta-5,7,22-trien-3β-ol. Administration of 3,-^{14}C-sitosterol to Digitalis lanata[49] plants has resulted in radioactive stigmasterol and dehydrogenation of saturated sterol side chains at C22 has been observed in clam, fungus and protozoan.[49]

The 9β,19-cyclopropane ring of cycloartenol hinders demethylase attack at the C14 position and consequently loss of one of the C4 groups occurs first. Loss of the C14 group is facilitated by a $\Delta^{7,8}$ bond which occurs when the cyclopropane ring is opened. Lanosterol possesses no cyclopropane ring but does have a Δ^{8} bond and during the biosynthesis of cholesterol in animal tissues the C14 group is the first to be lost.

Fig I.3 Possible routes for phytosterol synthesis

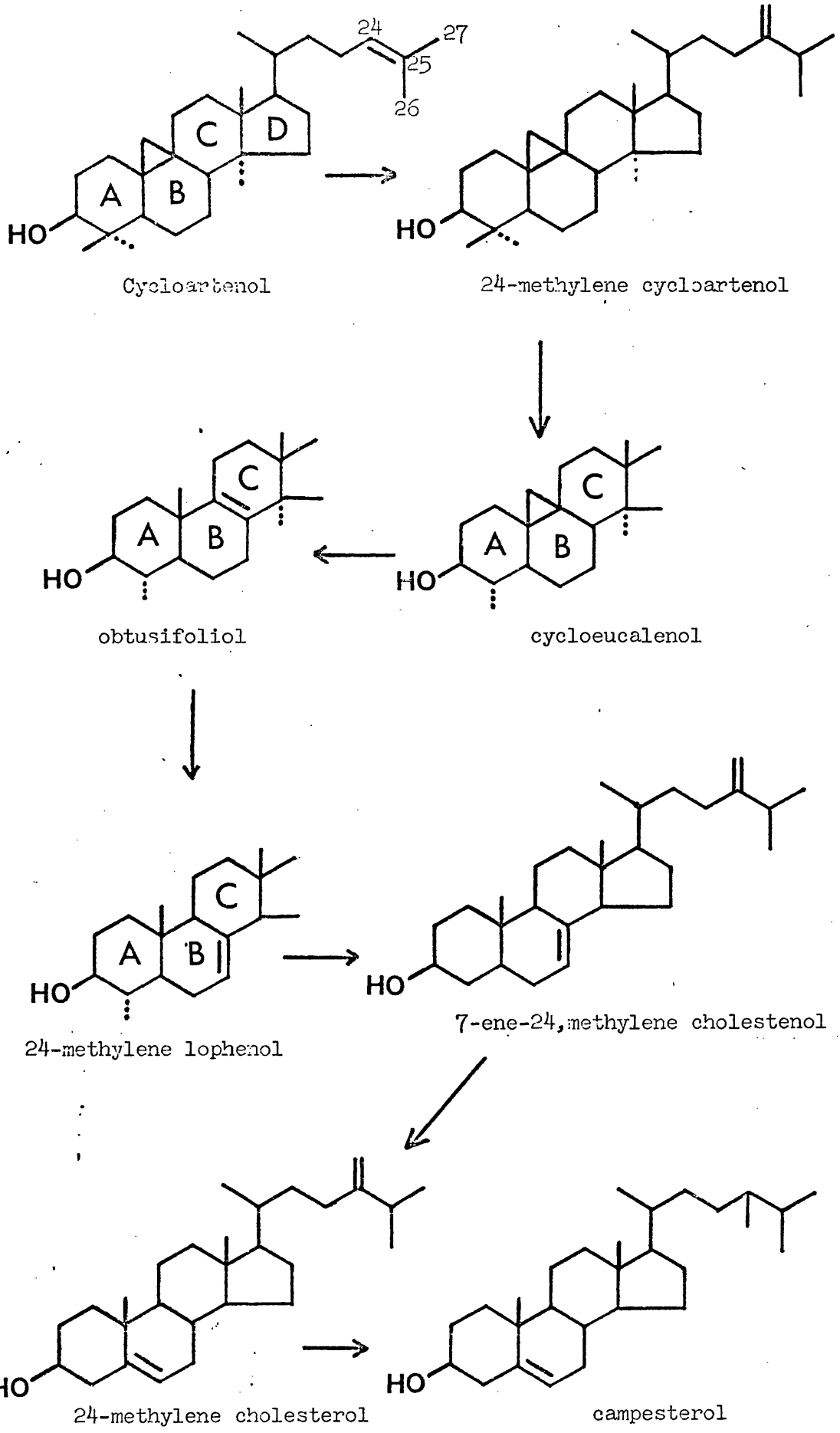
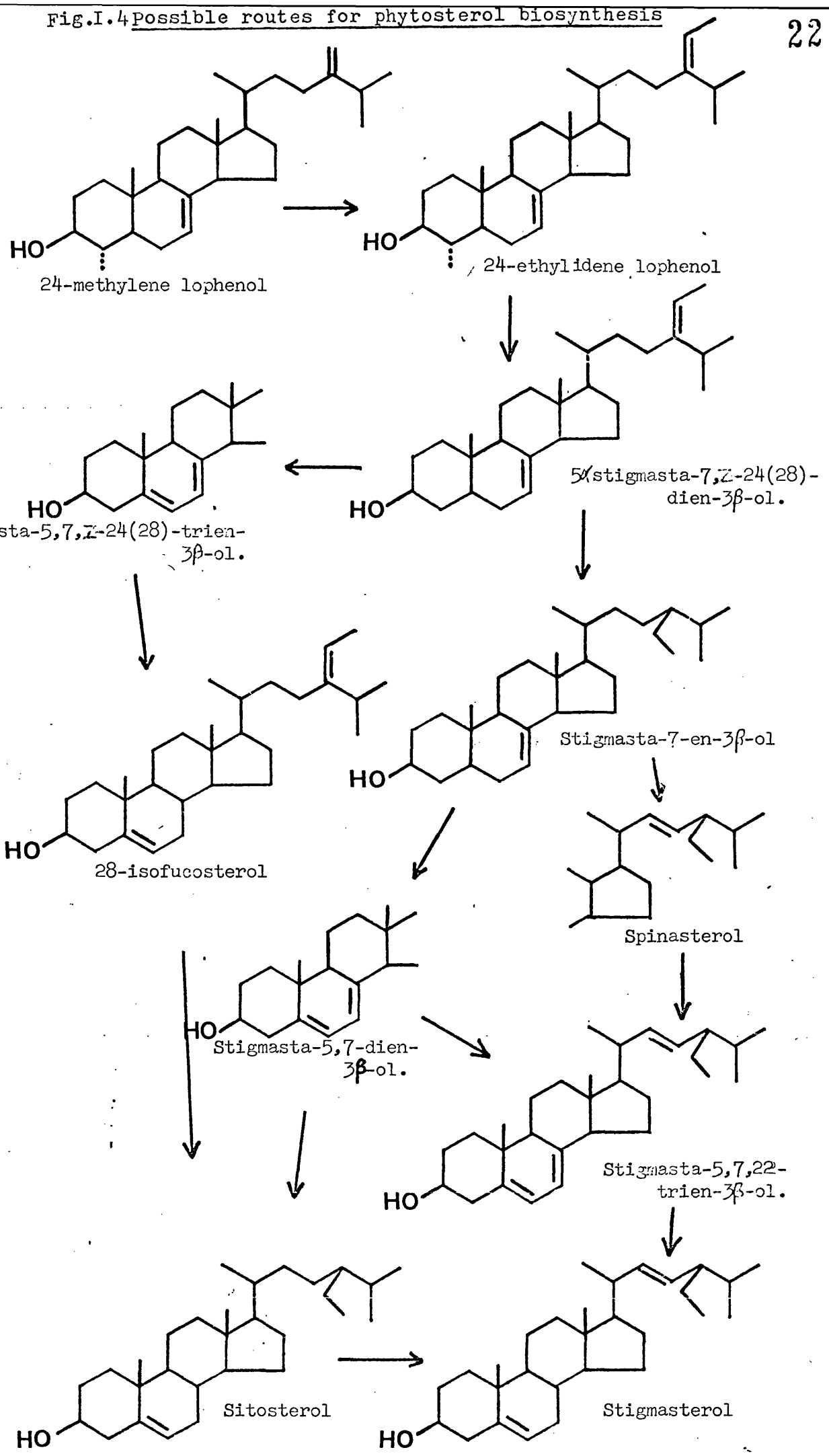


Fig.I.4 Possible routes for phytosterol biosynthesis



The routes outlined provide only a guide to the possible pathways of sterol biosynthesis. Goad and Goodwin [33] stated that any lack of specificity of the enzymes responsible for C24-alkylation, C4 C14 demethylation, or opening of the 9β,19-cyclopropane ring, could give diverging and converging pathways. Evans [34] examined the sterols of Digitalis purpurea and found pairs of compounds such as obtusifoliol and 4α-methyl zymosterol which suggested that methylation of the side chain was also non-specific and a non-linear process. It is probable that the relative importance of different routes varies greatly from species to species and depends on the enzyme specificity in different plants.[32]

THE BIOSYNTHESIS OF SAPOGENINS

Ehrhardt et al [50] isolated cycloartenol and 24-methylene cycloartenol from Dioscorea composita tissue cultures and postulated that cycloartenol might be the precursor of cholesterol and steroidal sapogenins in plants. The incorporation of 24-tritiated cycloartenol in diosgenin, yonogenin and tokorogenin in tissue cultures of Dioscorea tokoro [51] confirmed this hypothesis.

In 1965 Bennett and Heftmann [52] obtained incorporation of 4-^{14}C, cholesterol into diosgenin by Dioscorea spiculiflora. Using 4-^{14}C, 25-^{3}H-cholesterol with Dioscorea floribunda Joly et al [53] obtained diosgenin whose labelling indicated the incorporation of the intact cholesterol molecule into diosgenin. Stohs et al [54] obtained equal incorporation of both 4-^{14}C and 26-^{14}C, cholesterol into diosgenin in Dioscorea deltoidea tissue cultures and also concluded that the entire cholesterol

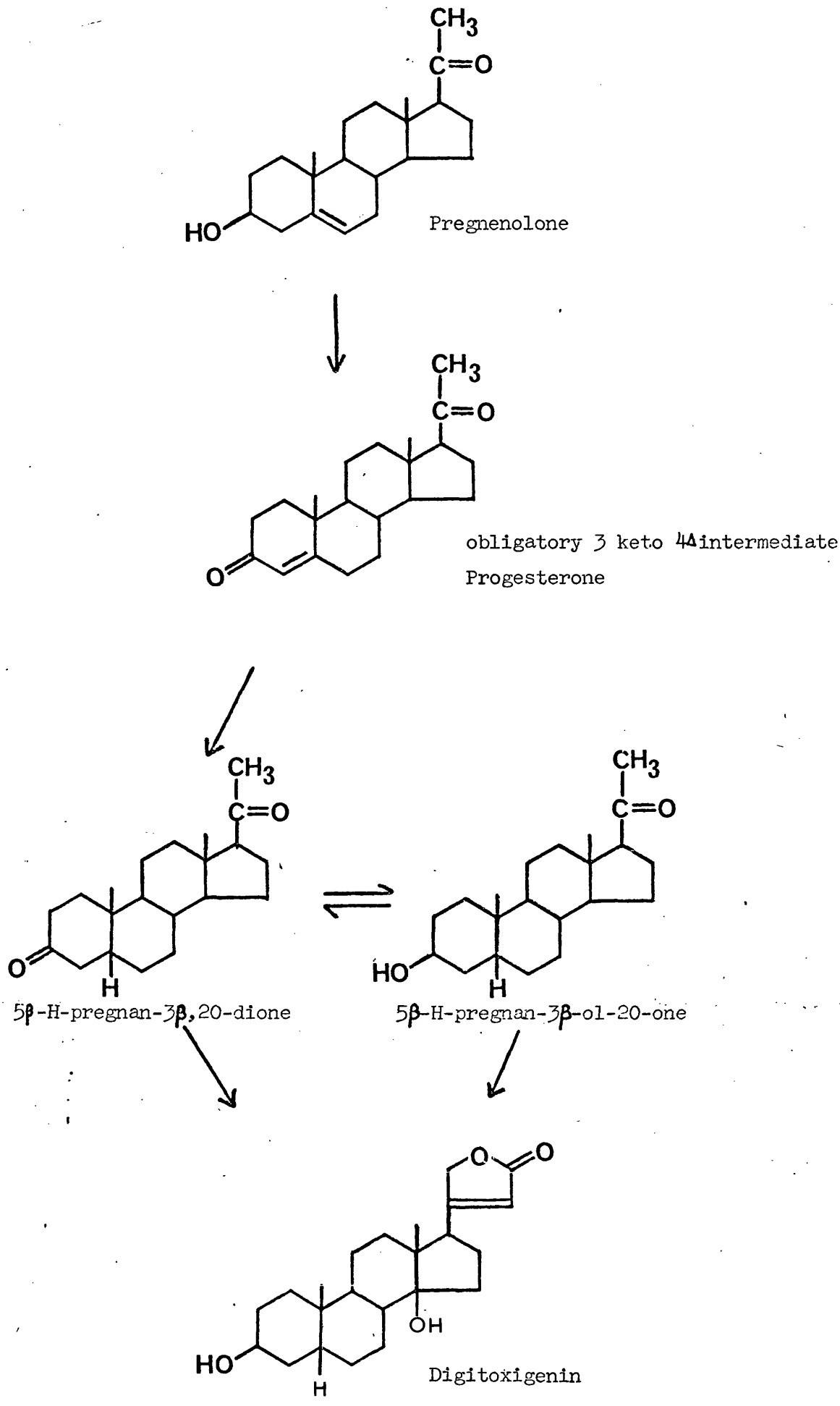
molecule was involved. The role of cholesterol as a key intermediate in the biosynthesis of spirostanols was thus accepted.

Both spirostanols and cardenolides are biosynthesised by species of Digitalis and the cardenolide precursor has been shown to be pregnenolone.[55] To produce molecules such as digitoxigenin, hydroxylase enzymes which introduce hydroxyl groups at C-12, C-14 and C-16 must be present.[56] Hydrogenation of pregnenolone to form an A/B cis derivative also occurs and this is only possible via a 3-keto $\Delta 4$ unsaturated derivative, progesterone.[55 56] The 3-keto and 3β-hydroxy derivatives are easily interconvertible in the plant, e.g. 5β-H-pregnan-3β-ol-20-one and 5β-H-pregnan-3β,20-dione in Digitalis plants and both are converted to digitoxigenin.[57] Fig.I.5

Tschesche[56] postulated a similar 3-keto stage in the formation of the A/B trans spirostanol compounds. Stohs and El-Olemy [58] found that tissue and leaf culture homogenates of Cheiranthus cheiri readily metabolised 4-^{14}C cholesterol to cholest-4-en-3-one, and suggested that the formation of this compound may be the first step in the synthesis of cardenolides. In Digitalis this same compound is converted to spirostanols via cholestan-3-one and cholestan-3β-ol.[56]

Marker and Lopez[59] first postulated that steroidal sapogenins could exist in plants in a form in which the side chain was held open by glycoside formation. In such compounds the F ring would be open and mild hydrolysis would yield the open chain aglycone whilst stronger hydrolysis would close the ring to form a spirostan aglycone. Joly et al[60] treated Dioscorea

FigI.5 The hydrogenation of pregnenolone via a 3 keto 4Δ intermediate



floribunda with 4-^{14}C-cholesterol and isolated the open chain glycoside Δ^5-furostene-3β,22,26-triol-3β-chacotrioside 26-β-D glucopyranoside. A leaf homogenate of the same plant[6I] converted this compound to labelled dioscin by F ring closure and subsequent hydrolysis of dioscin yielded diosgenin.

Bennett et al [62] have reported that 26-^{14}C,26-hydroxy cholesterol was converted to diosgenin by Dioscorea floribunda and suggested that oxygenation at C-26 may be the first step in introducing oxygen into side chain of cholesterol. Takeda's[63] work, in which a higher incorporation of 26-^{3}H,26-hydroxy-cholesterol than 16α-^{3}H,16β-hydroxycholesterol in sapogenins was obtained, supported this view. Tomita and Uomori[5I] obtained incorporation of both 26-^{3}H,16,26,-dihydroxy cholesterol and 22,16-^{3}H,16,22,26-trihydroxy cholesterol into diosgenin in Dioscorea tokoro tissue cultures. A pathway for the biosynthesis of diosgenin from cholesterol is given in Fig. I.6

Heftmann et al have shown that interconversion of 25R-(diosgenin) to 25S- (yamogenin) sapogenins or, vice versa, cannot occur in vivo. Takeda[63] postulated that the 25R-sapogenin was derived by hydroxylation of the cis-carbon at C-26 of cycloartenol and 25S-sapogenin by hydroxylation of the trans-carbon at C-27 (Fig. I.7). All other stages of the biosynthesis of the two epimers would then be the same.

FigI.6 Summary of the data on the biosynthesis of diosgenin

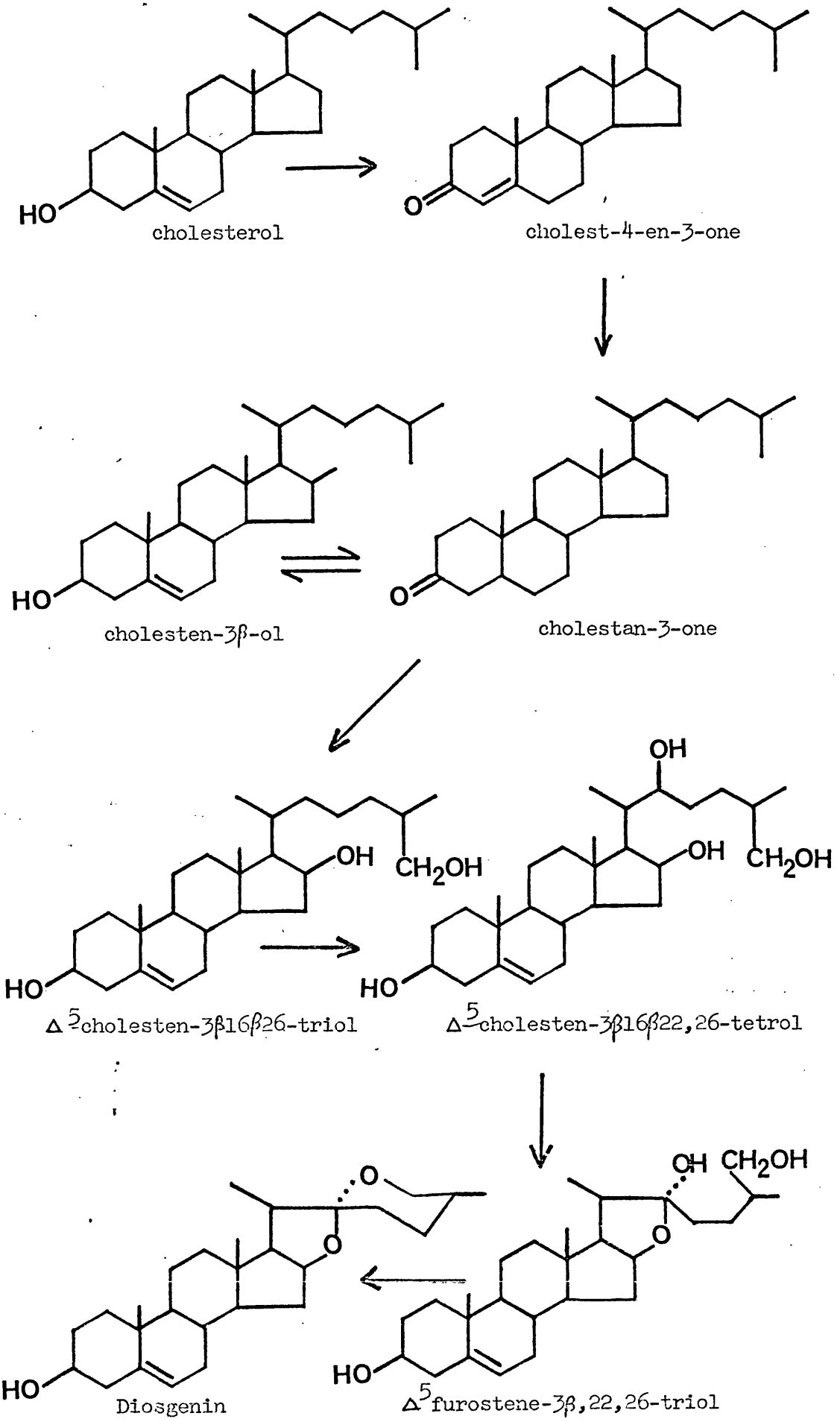
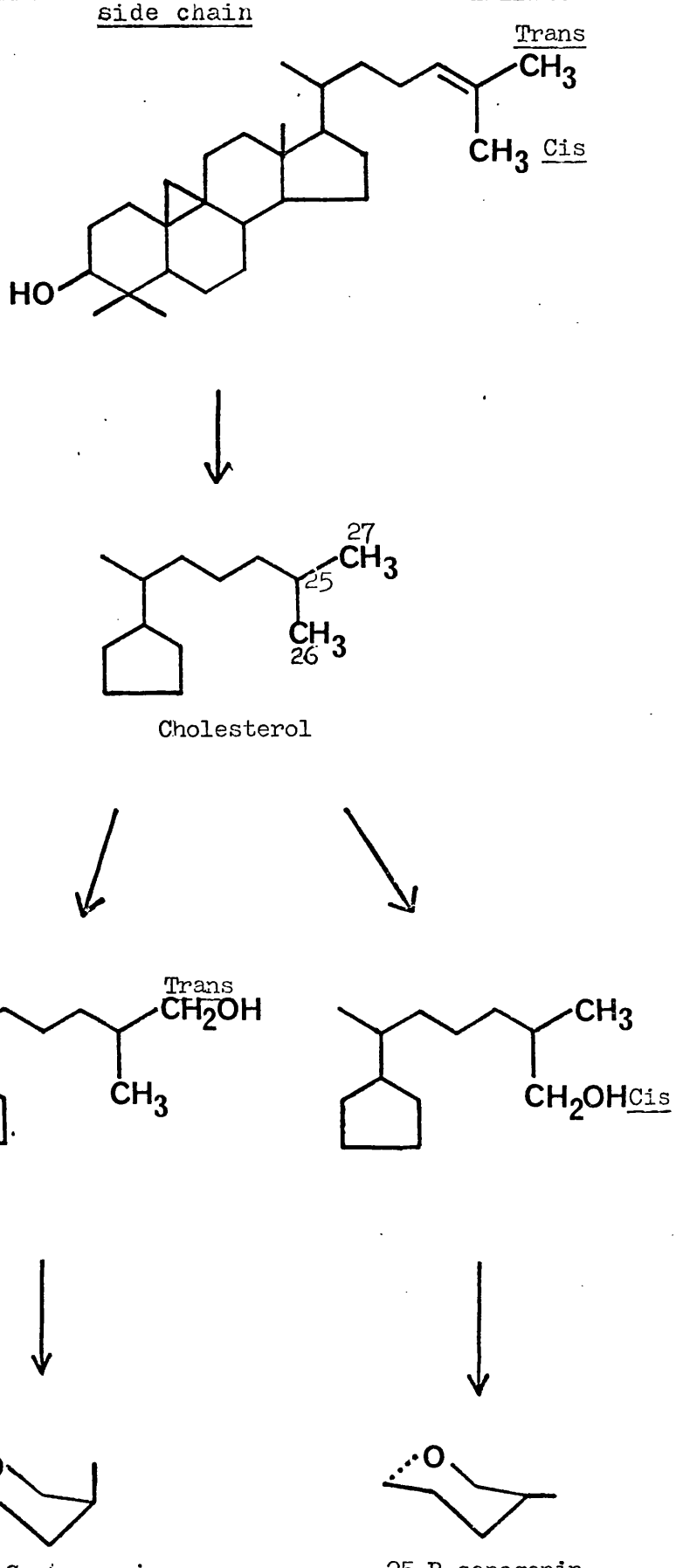


Fig.I.7 The hydroxylation of the cycloartenol side chain



REFERENCES

PART 1 - INTRODUCTION

1. Street, H.E. Plant tissue and cell culture, 1973, pp 1-30, Ed. H. E. Street, Blackwell Press,Oxford.

2. Haberlandt, G. Sber. Akad. Wus. Wien, 1902, 111, 69-92.

3. White, P.R. Plant Physiol. Lancaster,1934, 9, 585-600.

4. Gautheret, R.J. Acad. Sci. Paris, 1938, 206, 125-127.

5. Gautheret, R.J. Acad. Sci. Paris, 1939, 208, 118-121.

6. Caplin, S. and Steward, F. Am. Bot., 1952, 16, 491-504.

7. Miller, C., Skoog, F., Okumera, F., von Saltza, M. and Strong, F. J. Am. Chem. Soc., 1952, 78, 1375-1380.

8. Muir, W.M., Hildebrandt, A. and Riker, A.J. Science,N.Y., 1954, 119, 877-878.

9. Melchers, G. and Bergmann, L. Ber. dt. bot. Ges., 1959, 71, 459-73.

10. Cocking, E.C. Nature Lond., 1960, 187, 927-929.

11. Galston, A.W. and Davies, P. Science, 1969, 163,1288-1297.

12. Haagen-Smit, Am. J. Bot. 29, 500

13. Key, J.L., Plant Physiol., 1964, 39, 365-370.

14. Masuda, Y. and Kamisaka, S. Plant and cell physiol.,1969, 10, 79-86.

15. Nooden, L.D. and Thimann, K.V. Plant Physiol., 1965, 40, 193.

16. Merkys, A., Putrimas, A. and Marciukaitis, A. Flora, Abt. A, 1969, 160 (5), 516-532.

17. Parkash, V. J. Sci. & Ind. res., 1972, 31 (1), 35-38.

18. Brandes, M. and Kende, H. Plant Physiol., 1968, 43, 827.

19. Wilson, S.B., King, P.J. and Street, H.E. J. exp. Bot. 1971, 21, 177-207.

20. Klein, R.M. Econ. Bot., 1960, 14, 286-289.

21. Puhan, Z. and Martin, S.M. Progressin industrial microbiology, 1971, pp 14-39. Ed. D. J. D. Hockenhull. Churchill J.& A. London.

22. Turner, T. The Pharmaceutical Journal, 1971, May, 341-344.

23. Buchner, S. and Staba, E.J. J. Pharm. Pharmac., 1964, 16, 733-737.

24. Hardman, R. Trop. Sci., 1969, 11 (3), 196-228.

25. Kaul, B. and Staba, E.J. Lloydia, 1968, 31 (2), 171-179.

26. Mehta, A. and Staba, E.J. J. Pharm. Sci., 1970, 59 (6), 864-65.

27. Nickell, L. G. Advanced Appl. Microbiol., 1962, 4, 213.

28. Ehrhardt, J.D., Hirth, L. and Ourisson, G. Compt. Rend., 1966, 262, 809.

29. Fazli, F.R.Y. and Hardman, R. Phytochem., 1971, 10, 2497-2503.

30. Fazli, F.R.Y. and Hardman, R. Trop. Sci., 1968, 10, 66.

31. Hardman, R. to National Research Development Corporation, (London), Brit.Pat. 1,136,626, 1968.

32. Jefferies, T.M. Ph.D. Thesis, Bath University, 1973.

33. Goad, L.J. and Goodwin, T.W. The Biosynthesis of plant sterols. Progress in phytochemistry, 1972, Vol.3, pp.113-198. Interscience, London.

34. Evans, F.J. J. Pharm. Pharmac., 1972, 24, 227-234.

35. Cornforth, J.W., Cornforth, R.H., Popjak, G. and Yengoyan, L. J. Biol. Chem., 1966, 241, 3970-3987.

36. J. Lipid Res., 1959, 1, 3-28. Cornforth, J.W.

37. Loomis, W.D. and Battaile, J. Biochim. Biophys. Acta, 1963, 67, 54-63.

38. Graebe, J.E. Phytochem., 1968, 7, 2003-2020.

39. Capstack, R., Rosin, N.L., Blondin, G.A. and Nes, W.R. J. Biol. Chem., 1965, 240, 3258-3263.

40. Arch. Biochem. Biophys., 1963, 102, 26-32. Beeler, D.A. Anderson, D.G., & Porter, J.W.

41. Corey, E.J., Russey, W.E. and Ortiz de Montellano, P.R. J. Am. Chem. Soc., 1966, 88, 4750-4751.

42. Benveniste, P. and Massey-Westrop, R.A. Tetrahedon Lett., 1967, 37, 3553-3556.

43. Benveniste, P., Phytochem., 1968, 7, 951-953.

44. Ehrhardt, J.D., Hirth, L. and Ourisson, G. Compt. Rend., 1966, 262, 809-811.

45. Rees, H.H., Goad, L.J. and Goodwin, T.W. Biochem. J., 1968, 107, 417-426.

46. Goad, L.J. Terpenoids in plants, 1967, 159.(Ed. J. B. Pridham), Academic Press, London.

47. Baisted, D.J. Phytochem., 1969, 8, 1697-1703.

48. Devys, M., Alcaide, A. and Barbier, M. Bull. Soc. Chem. Biol., 1968, 50, 1751.

49. Bennett, R. and Heftmann, E. Steroids, 1969, October, 403-407.

50. Ehrhardt, J.D., Hirth, L. and Ourisson, G. Phytochem., 1967, 6, 815-821.

51. Bennett, R.D. and Heftmann, E. Phytochem., 1965, 4, 577-586.

52. Tomita, Y. and Uomori, A. Chem. Commun., 1971, 284.

53. Joly, R.A., Bonner, J., Bennett, R.D. and Heftmann, E. Phytochem., 1969, 8, 1709-1711.

54. Stohs, S.J., Kaul, B. and Staba, E.J. Phytochem., 1960, 8, 1679-1686.

55. Sauer, H., Bennett, R. and Heftmann, E. Phytochem., 1967, 6, 1521-1526.

56. Tschesche, R. Proc. R. Soc. London B., 1972, 180, 187-202.

57. Bennett, R., Sauer, H. and Heftmann, E. Phytochem., 1968, 7, 41.

58. Stohs, S.J. and El-Olemy, M.M. J. Steroid Biochem., 1971, 2, 293-298.

59. Marker, R. and Lopez J. J. Am. Chem. Soc., 1947, 69, 2386-2392.

60. Joly, R., Bonner, J., Bennett, R. and Heftmann, E. Phytochem., 1969, 8, 857-859.

61. Joly, R., Bonner, J., Bennett, R. and Heftmann, E. Phytochem., 1969, 8, 1445-1447.

62. Bennett, R., Heftmann, E. and Joly, R. Phytochem., 1970, 9, 349-353.

63. Takeda, K. Progress in phytochemistry, Vol.3, 1972,pp.287-334. Interscience, London.

PART II CHAPTER I

THE INDUCTION OF FENUGREEK TISSUE CULTURES

THE SELECTION OF A BASAL MEDIUM FOR FENUGREEK TISSUE CULTURES

The basis of all nutrient media is a mixture of mineral salts combining the essential macro and micro elements, together with a source of carbon, which is normally a sugar and usually sucrose. Supplements included in such media are vitamins, amino acids, auxins and related growth regulators, gibberellins, a chelate such as E.D.T.A., kinetin or other cytokinin and sometimes a natural organic extract. The use of solutions of natural origin such as coconut water, yeast extract and casein hydrolysate is widespread, although the desirability of fully defined media has led to decrease in their use. Coconut water is still often used in the initiation of new cultures where the nutrient requirement is not known and attempts have been made to standardise the effects of coconut water by biological assay and market it in the freeze-dried form.

(1) Carbohydrates

A number of carbohydrates including mono, di and polysaccharides have been studied as carbon sources in plant tissue culture. Such a source has always been found necessary in cultures, so far reported, because of the inability of tissues to photosynthesise sufficiently to maintain basal metabolism. Sunderland [1] found that withdrawal of sugar from tissue cultures of Oxalis dispar resulted in loss of fresh weight increase and eventual death.

Of the many compounds tested as a carbon source sucrose is the most successful, although sorbitol and mannitol have been used in some cultures. [2] Fructose has been beneficial

in some cases and detrimental in others.[3] Dextrose, although a good carbon source, has generally given poorer results than sucrose. Risser and White[4] found that dextrose was the only suitable substitute for sucrose in spruce tissue culture while fructose, mannose and raffinose only maintained growth at 50% of that on sucrose.

(2) Macro nutrients

Actively growing plant tissues require a continuous supply of inorganic elements and the composition of the mineral salts must be taken into account. Apart from carbon, oxygen and hydrogen the essential macro elements are nitrogen normally added as nitrate; phosphorus added as phosphate; sulphur as sulphate and potassium, present as the major cation. Magnesium, calcium, sodium and chloride are also essential, but in smaller amounts. Several mixtures of inorganic macro nutrients are in common use and Street[5] has compared those in most common use in the following table. (Table II.I)

TableII.I

Comparison of media macro-nutrients used in tissue culture

(milligrammes per litre)

Constituent	Miller	Nitsch and Nitsch	White	Hildebrandt Riker and Duggar	Murashige and Skoog
KCl	750	1500	65	65	
$NaNO_3$	600				
$MgSO_4 7H_2O$	250	250	720	180	370
$NaH_2PO_4 H_2O$	125	250	16.5	33	
$CaCl_2 2H_2O$	75				440
KNO_3		2000	80	80	1900
$CaCl_2$		25			
Na_2SO_4			200	800	
NH_4NO_3					1650
KH_2PO_4					170
$Ca(NO_3)4H_2O$			300	400	

(3) Micro nutrients

Most media contain trace amounts of certain inorganic nutrients. Such substances are often present as contaminants of macro nutrients, but they are added to all the standard media. The most common additives are boron, copper, iodine, iron, manganese, molybdenum and zinc. It is hard to demonstrate the effects of micro nutrient deficiency because of the difficulty of eliminating them from the macro nutrients used. Boron deficiency has been shown in callus and root cultures of Helianthus annus and it was manifested as a decrease in growth rate and lignification.[6] Street[5] recommends that iron should be added as sodium ethylenediamine tetra acetate to ensure a uniform release over a wide pH range.

(4) Vitamins

The number of vitamins included in published formulations varies greatly from two or three[7] to a large number.[8] Among the most common are p-aminobenzoic acid, ascorbic acid, biotin, choline, cyanocobalamine, folic acid, m-inositol, nicotinic acid, pantothenic acid, pyridoxine, riboflavine and thiamine. Studies with root cultures have revealed that some vitamins may be synthesised in growth.[9]

(5) Amino acids

The maintenance of callus or suspension cultures may require the presence of selected amino acids. Cultures obtained from Ginko pollen were found to require arginine[10] and asparagine was found to be highly specific in the stimulation of maize endosperm growth.[11] Steward and Shantz[12] reported that tryptophan with coconut water may

stimulate growth and Skoog and Miller[13] observed an enhancement of cytokinin induced organogenesis in media supplemented with tyrosine. Glycine has been included in Murashige and Skoog's medium[7] and cysteine was used by White.[14] A complex mixture of amino acids may stimulate or inhibit growth, and according to Harris[15 16] some amino acids may antagonise one another. Coconut water contains a wide range of amino acids but in sub-optimal concentrations for an effect on growth.[17] Yeast extract and casein hydrolysate have been used as sources of amino acid. Stimulation of growth caused by the addition of amino acid normally only occurs if an inorganic nitrate source is present in the medium.

(6) Growth regulating substances

Few excised tissues are capable of maintaining sustained callus growth in the absence of added growth factors. Street[5] has placed plant tissues in four categories.

(i) Tissues which require only an auxin or related growth regulator

(ii) Those that require only a cytokinin

(iii) Those that require both an auxin and a cytokinin

(iv) Tissues which will only respond to media containing complex natural extracts.

Most tissue culture systems include both auxin and cytokinin in the medium and a natural extract such as coconut water is sometimes used.

The auxins and related growth regulators normally used are indole-3yl-acetic acid (IAA), naphthalene acetic acid (NAA) and 2,4-dichlorophenoxyacetic acid (2,4-D). According to Kefford and Goldacre[18] plant growth substances such as

IAA, NAA and 2,4-D are correlative regulators which predispose cells to change, the exact nature of which is determined by other chemical regulators such as the gibberellins and cytokinins. Cytokinins are, by definition, naturally occurring substances and are present in extracts from green plants but few of the natural cytokinins have been characterised. Kinetin has been widely used to initiate and maintain growth in callus cultures and is particularly active as an interactant with IAA or 2,4-D in tobacco pith cultures.[7] The plant growth regulator requirements of different species vary greatly in type and concentration for optimum growth, organogenesis or biosynthesis of specific products.

MURASHIGE AND SKOOG'S TOBACCO TISSUE CULTURE MEDIUM

(1) Composition of the medium

Of the commonly used media many are based on White's original macro-nutrient formulation, designed for excised root cultures. Hildebrandt, Riker and Duggar[19] improved the medium for specific tissues and reported two new formulations for the optimal growth of hybrid tobacco callus and sunflower crown gall devised by a triangulation method. Heller[7] made detailed studies of the mineral requirements of cultures of carrots and virginia creeper and the medium devised gave two or three times greater yields than White's original medium. Murashige and Skoog[7] found that none of these available media provided sufficient amounts of some of the essential elements for optimum growth of Winsconsin 38 Nicotiana tabacum cultures. They carried out experiments using White's modified nutrient solution, supplemented with myo-inositol and edamin, as a

reference medium, to devise a better medium. Preliminary tests showed that doubling or quadrupling the levels of inorganic constituents increased the yield of tissue. When each element was increased to four times the original concentration all except sulphur gave improved yield and increasing all the salts gave a greater effect than increasing any one element. White's medium was found to be particularly deficient in nitrogen and potassium.

Iron is a critical requiremcnt for vigorous and prolonged growth and Murashige and Skoog supplied it as NaFe-E.D.T.A. chelate. Alterations in the range of concentrations of copper, iodine and molybdenum had no effect on tissue growth. The pH of the medium was adjusted to 5.7 or 5.8 with 1N hydrochloric acid or potassium hydroxide. This value was selected because it was found that more acid or basic media tended to drift towards this region during heat treatment. A pH of 5.8 was found to keep all the salts in soluble form and was low enough to allow growth and differentiation of the tissues.

Brain (1958) and Steinhart (1962)[7] had found that addition of myo-inositol promoted growth when other conditions were favourable and Murashige and Skoog included it in the medium. No other changes were made in the vitamin content of the White's medium.

Carbohydrate was supplied in the form of sucrose and a concentration of 3% was found to be optimal for growth. Agar was recommended at 1% w/v to give a suitably moist but rigid medium.

Murashige and Skoog[7] found that the optimal requirement for either auxin or cytokinin increased with an increase in the other. The effect of a given combination at intermediate

concentrations was found to vary from one experiment to another. Specific levels and ratios of IAA and kinetin affected the form and texture of the callus, the development of plant organs and the vigour and longevity of the cultures. For the continuous growth of firm healthy callus over a period of two years 0.2 mg/litre of kinetin and 2.0 mg/litre of IAA were reported to be satisfactory. Higher levels of both auxin and kinetin were required for long lasting subcultures (2 to 6 months) and concentrations as high as 30 mg/litre of IAA and 6 mg/litre of kinetin were employed. NAA and 2,4-D were tested as substitutes for IAA and, because of the lower rates of inactivation, levels of 0.05 to 0.2 mg/litre were satisfactory for callus growth. The medium was specifically designed to provide excess inorganic nutrients so that unintentional addition of salts, in plant extracts being screened for growth promoting activity, would not effect the tissue growth response.

Murashige and Skoog's medium[7] was thought to provide a good basic medium for the initiation of tissue cultures from most plant sources although modification might be necessary for optimum growth. Preliminary work[20] had shown that it was possible to initiate and maintain tissue cultures of Fenugreek on this medium for a period of several months with subculturing at 30 day intervals, when a supplement of 10% v/v of coconut water was included and IAA replaced with 10 ppm NAA. The composition of Murashige and Skoog's revised tobacco medium (MS medium) is shown in TableII.2. Stock

solutions of the medium constituents were prepared, see TableIII.I,and fresh medium was prepared on each occasion that it was required.

Table II.2

The composition of Murashige and Skoog's Tobacco Medium.

Mineral Salts

Major Elements			Minor Elements		
Salts	mg/l	mM	Salts	mg/l	mM
NH_4NO_3	1650	41.2	H_3BO_3	6.2	100
KNO_3	1900	18.8	$MnSO_4 4H_2O$	22.3	100
$CaCl_2 2H_2O$	440	3.0	$ZnSO_4 4H_2O$	8.6	30
$MgSO_4 7H_2O$	370	1.5	KI	0.83	5.0
KH_2PO_4	170	1.25	$NaMoO_4 2H_2O$	0.25	1.0
Na_2EDTA	37.3	Na 0.20	$CuSO_4 5H_2O$	0.025	0.1
$FeSO_4 7H_2O$	27.8	Fe 0.10	$CoCl_2 6H_2O$	0.025	0.1

Organic Constituents

Sucrose	30 G/l	Agar	10G/l
Edamin (opt.)	1 G/l	Myo-inositol	100 mg/l
Glycine	2.0 mg/l	Nicotinic acid	0.5 mg/l
Indolacetic acid	1-30 mg/l	Pyridoxin HCl	0.5 mg/l
Kinetin	0.04-10 mg/l	Thiamine HCl	0.1 mg/l

(2) Experiment of study change in pH of media after autoclaving

Murashige and Skoog[7] reported that the effect of autoclaving of both acid or basic media was to shift the pH to a value nearer to 5.7-5.8. A preliminary experiment was carried out to estimate the change in pH which occurred when MS medium was supplemented with 10% v/v coconut water and autoclaved at 15 psi for 15 mins. It was found that our coconut water tended to buffer the medium at pH 5.25 and quite large amounts of 1N, potassium hydroxide or hydrochloric acid solutions were required to change the pH prior to autoclaving.

A series of media were prepared over a range of pH from 5.2 to 6.4 and the pH of the media measured before and after autoclaving. The results, TableII.3 , do not agree with the results obtained by Murashige and Skoog. In the presence of coconut water the pH of all the media fell on autoclaving. A linear relationship was obtained between the initial and final pH from pH 5.2 to 6.6, Fig.II.I The buffering effect of the coconut water could explain the fall in pH in all cases except the trial media at 5.2 which fell to 5.1. A tobacco tissue culture pH of 5.7-5.8 had been used by Murashige and Skoog. For work on Fenugreek the pH of the medium before autoclaving was adjusted to 6 which gave a pH of 5.65 after autoclaving.

Several trial samples were prepared at pH 6 and autoclaved and the pH of the autoclaved media were between 5.6 and 5.7

Table II.3

The effect of autoclaving on the pH of Murashige and Skoogs medium supplemented with 10% coconut water

pH before autoclaving	pH after autoclaving
5.20	5.11
5.39	5.23
5.64	5.39
5.89	5.58
5.99	5.65
6.24	5.79
6.43	5.93
6.63	6.07
6.80	6.00

Slope = 0.6727

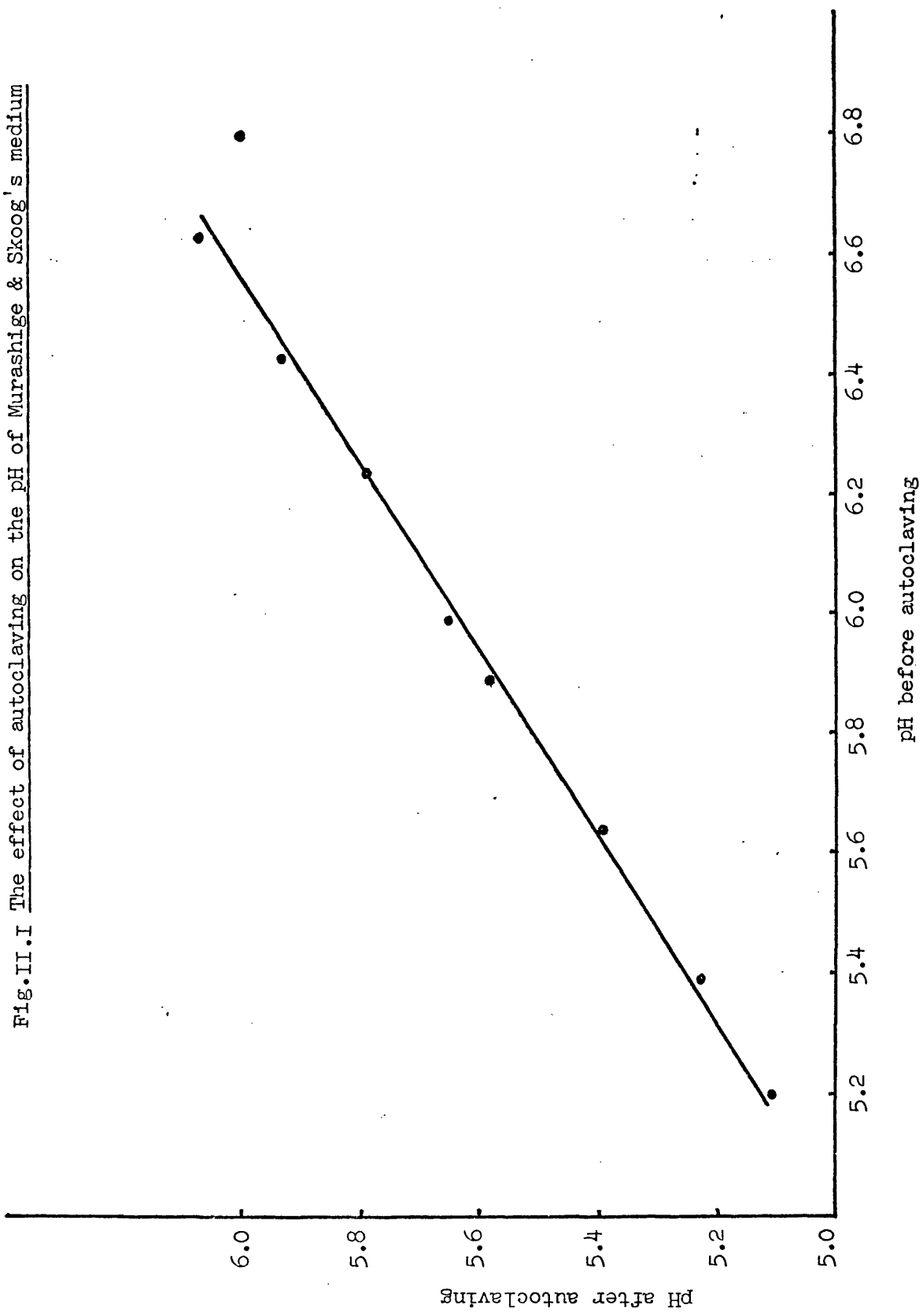


Fig.II.I The effect of autoclaving on the pH of Murashige & Skoog's medium

SEED SURFACE STERILISATION

The induction of a callus from a plant part occurs when a sterile explant is brought into contact with a nutrient medium known to induce and support cell division. In the case of seed, the sterile explant is acquired by surface sterilisation with a chemical reagent. After removal of the reagert with sterile water the seed can either be germinated, and the required part of the sterile seedling removed, or the seed can be placed on the medium, in which case, the callus is formed from mixed cells of the whole seed. The cultures used in the experiments in this thesis were induced from isolated cotyledons from sterile seedlings.

Several reagents have been used for the surface sterilisation of plant tissue explants, including bromine water and hydrogen peroxide.[5] An experiment was performed to see if these reagents would be suitable for the surface sterilisation of commercial Fenugreek seed, which was subject to heavy fungal spore contamination. A 20 seed sample of Ethiopian seed RH.2602, which was used only in preliminary work, was immersed in bromine water for two minutes, aseptically transferred to 10 volume hydrogen peroxide for ten minutes and washed with two quantities of sterile water. The seeds were placed on sterile Murashige and Skoog's (MS) medium, containing no auxin, and incubated for five days at 25°C. MS medium was used to provide favourable conditions for the growth of any fungal contaminants present on the seed. After five days incubation, 16 of the seeds showed signs of fungal infection.

Toplin and Gaden[21 22] used a 1% v/v solution of β-propiolactone as a sterilising agent for liquid media. In aqueous

solution this compound was unstable with a half life of 30 minutes at 50°C. A 20 seed sample of Ethiopian variety RH.2602 was immersed in a 1% v/v aqueous solution of β-propiolactone for 30 minutes at 50°C. The seeds were washed twice with sterile distilled water and incubated on MS medium as before. After five days only two seeds showed signs of contamination and 17 of the seeds had germinated. The seedlings were examined after seven days and five cotyledons showed signs of damage by the sterilising agent. Small areas of the normally yellow cotyledons were brown and appeared to be dead. The experiment was repeated with a 20 seed sample of the Moroccan seed RH.2336. All the seeds germinated and only two cotyledons showed signs of damage. No fungal contamination was detected after seven days incubation. The use of a 1% β-propiolactone solution for the surface sterilisation of seed was adopted as a routine procedure.

THE INDUCTION OF CALLUS CULTURES FROM STERILE COTYLEDONS

A ten seed sample of RH.2336 variety was surface sterilised and incubated on sterile distilled water containing 1% v/v of agar in the dark at 25°C. The cotyledons were removed from the seedlings and transferred to MS medium in which the IAA had been replaced by 10 ppm NAA and kinetin replaced by 10% v/v coconut water. Five cultures were grown in continuous light and five in total darkness at 25°C ± 1°C. Undifferentiated callus first became apparent at the cut surfaces of the cotyledons after 14 days. Cotyledons exposed to the light became green after three days and the callus cells induced, retained the ability to produce chlorophyll. The

callus tissue was removed after 30 days and sub-cultured onto fresh medium. The cultures continued to grow in an undifferentiated state in both light and dark conditions. High concentrations of auxin have proved inhibitory to chlorophyll formation in some cultures. Sunderland[I] found that 1 ppm 2,4-D or 10 ppm NAA inhibited chlorophyll formation in Oxalis dispar cultures, but Fenugreek cultures grown in the light with 10 ppm NAA were not similarly affected. The tissue produced by cultures grown in the dark was pale yellow or white and became brown on ageing.

PART II CHAPTER II

THE DETERMINATION OF THE OPTIMUM CONDITIONS FOR FENUGREEK TISSUE CULTURE GROWTH

PLANT GROWTH REGULATORS IN TISSUE CULTURES

The work of Murashige and Skoog [7] showed that the specific levels and ratios of IAA and kinetin affected the callus vigour, texture and longevity, and the development of plant organs from tissue cultures. The conditions required for the successful initiation and maintainence of cultures vary with species of plant and organ of origin. It is necessary to determine experimentally the optimum concentrations of growth regulators for a desired tissue response. The concentrations and ratio of auxin and cytokinin which produce the most vigorous growth of a tissue culture may not stimulate, and may even inhibit, the biosynthesis of secondary products characteristic of the plant of origin. In the case of Atropa belladonna cultures a change in hormonal balance which induced root development in a culture also resulted in alkaloid production in the previously alkaloid free tissue.[23]

Work carried out by Krikorian and Steward[24] showed that the type of auxin, as well as the concentration, can affect the secondary products biosynthesised. The alkaloids produced in Catharanthus tissue on two media containing 10% v/v coconut water and either 5 ppm 2,4-D,or 5 ppm NAA, differed. The levels of 2,4-D, kinetin and sucrose were found to affect the quantity of Klason lignin produced by Acer pseudoplatanus cultures.[25] Studies on anthocyanin accumulation by cultures of Haplopappus gracilis[26] also illustrated how the biosynthesis of secondary product can

be controlled by the level of growth regulator. Kaul, Stohs and Staba[27] found a 2,4-D concentration of 0.1 ppm optimal for diosgenin production by Dioscorea deltoidea tissue and suspension cultures.

Work on several species has also shown that the yield of characteristic secondary products from tissue cultures can vary with the cultures initiated by different workers. Dawson[28] reported that Nicotiana tabacum cultures lost the ability to produce nicotine after 28 days whilst Furuya[28] reported that 5-year-old stem callus cultures contained both nicotine and anabasine. It is clear that any work carried out on plant tissue cultures must begin with a study of the effect of auxin/cytokinin balance on the particular tissue callus culture initiated.

A series of experiments were, therefore, carried out to determine the growth response of the cultures (initiated from Fenugreek cotyledons) to different concentrations and to different ratios of growth regulators.

GROWTH RESPONSE OF FENUGREEK TISSUE CULTURES TO DIFFERENT COMBINATION OF GROWTH REGULATORS

(1) Growth response on induction medium

In these experiments 100 ml sterile plastic pots each containing 20 ml of medium and an innoculum of about 50 mg (weighed accurately) of tissue were used. The response of the tissue to these conditions was determined with the original medium, so that a suitable growing time would be determined for the quantity of medium to be supplied in subsequent experiments. Ten replicates were grown in continuous light and ten in continuous dark. The fresh weight of

each replicate was determined at intervals, by removing the individual tissues from their jars aseptically and weighing them in a tared empty sterile jar.

The growth index, calculated by the formula

$$\frac{\text{Final fresh wt.} - \text{Initial fresh wt.}}{\text{Initial fresh wt.}}$$

was determined for each culture, at the time periods shown, Tables II.4 to II.7

From graphs, Figs.II.2 and II.3, it can be seen that after an initial lag period of about 5 days, the tissue increased in fresh weight rapidly until 45-50 days in the light grown tissue and 55-60 in the dark grown tissue. The initiation of the rapid growth rate occurred earlier in the tissue grown in the light. The results show a linear growth phase between 20 and 35 days for light grown tissues and 30 and 60 days for dark grown cultures. Caplin[29], in a similar experiment, found that the variation in growth rate which occurred between replicates on a given medium increased with age. This was not the case in this experiment, and it can be seen that the 95% confidence interval for the population mean weight for each determination remained at approximately ± 20% throughout the growth period. The growth rate of the tissues grown in the light decreased after 40-50 days and that of the dark grown tissues diminished after 55 days.

Murashige and Skoog[7] chose a growth period of 28 days for media evaluation experiments because it extended past the linear growth phase. For Fenugreek tissue cultures grown in continuous light the linear growth phase finished after 35

Table II.4

Weight of tissue cultures grown in continuous darkness

DAYS OF GROWTH

1	5	20	30	40	50	60	70
.204	.359	.803	1.593	3.696	5.032	5.943	6.103
.163	.253	.899	1.968	3.523	4.667	6.988	7.081
.174	.306	.838	1.364	2.068	2.650	4.235	4.282
.048	.076	.189	0.279	0.751	1.068	1.751	1.981
.079	.079	.163	0.359	0.776	1.668	3.983	3.942
.108	.149	.290	0.651	1.089	1.513	2.363	2.450
.167	.241	.693	1.390	2.837	3.797	5.447	5.481
.081	.087	.198	0.523	1.194	2.050	4.395	4.891
.071	.091	.203	0.724	1.302	2.182	4.091	5.254
.145	.229	.732	1.586	3.087	4.155	7.535	7.810
1.24	1.87	5.01	10.43	20.32	28.78	46.73	49.27
.12	.18	.50	1.04	2.03	2.88	4.67	4.92

wt. in gms

y

$\bar{y}$

Table II.5

Growth indices of tissue cultures grown in continuous darkness

DAYS OF GROWTH

1	5	20	30	40	48	60	70	
0	0.76	2.94	6.81	17.12	23.66	28.13	28.9	
0	0.55	4.52	10.10	20.61	27.59	41.87	42.44	
0	0.76	3.82	6.84	10.88	14.23	23.33	23.61	
0	0.58	2.94	4.81	14.65	21.25	35.48	40.27	
0	0.00	1.06	3.54	8.82	20.11	49.42	48.90	
0	0.37	1.68	5.03	9.08	13.01	20.88	21.68	
0	0.44	3.15	7.32	16.98	21.73	32.57	32.82	
0	0.07	1.44	6.45	14.74	24.31	53.25	59.38	
0	0.28	1.85	9.99	18.35	29.73	56.70	73.01	
0	0.58	4.05	9.93	20.28	27.65	50.97	32.86	
	2.5	27.51	70.0	151.5	233.3	392.6	423.8	Σx
	0.25	2.75	7.00	15.15	23.33	39.26	42.38	$\bar{x}$
	4.4	88.1	534.4	2464.7	5260.3	16935.0	20404.5	Σx^2
	.20	.38	0.69	1.37	3.46	4.1	5.20	$S\bar{x}$
	.46	.85	1.58	3.09	3.95	9.29	11.76	$\pm$

Table II.6

Weights of tissue cultures grown in continuous light

1	10	20	26	33	40	50	60	DAYS
.043	.136	.351	.674	1.244	3.033	4.457	4.612	gms
.033	.231	.572	1.362	2.453	4.006	4.211	4.303	
.033	.317	.634	1.358	2.376	4.166	4.503	4.564	
.063	.400	.710	1.336	2.696	4.384	5.128	5.910	
.047	.317	.664	1.475	2.3723	3.474	3.341	3.187	
.047	.372	.687	1.410	2.723	3.521	3.731	3.952	
.040	.275	.605	1.336	2.338	3.266	3.665	4.210	
.040	.074	.185	0.672	1.492	2.561	3.505	4.271	
.071	.483	.866	2.158	3.644	4.560	4.574	4.587	
.054	.205	.641	1.170	1.896	3.170	3.929	4.580	
0.47	2.81	5.92	12.95	23.23	36.13	41.04	44.17	Σ x
0.05	0.28	0.59	1.29	2.32	3.613	4.10	4.417	$\bar{x}$

Table II.7

Growth indices of tissue cultures grown in continuous light

1	10	20	26	33	40	50	60	
0	2.16	7.16	14.67	27.93	69.53	103.65	106.25	
0	6.00	17.33	40.27	73.33	120.42	126.60	129.39	
0	8.61	18.21	40.15	71.00	125.24	135.45	137.3	
0	5.35	10.27	20.21	41.70	68.52	80.26	93.80	
0	5.74	14.13	30.38	49.46	72.91	70.10	67.81	
0	6.91	13.61	29.00	56.93	73.91	78.38	84.08	
0	5.87	14.12	32.40	57.45	81.50	91.50	105.20	
0	0.85	3.62	15.80	36.30	64.02	88.00	106.70	
0	5.80	11.20	29.40	50.33	64.15	64.35	64.54	
0	2.80	10.87	20.66	34.11	57.70	72.75	84.80	
0	50.1	120.5	273.9	498.4	797.9	911.0	979.8	Σx
0	5.01	12.05	27.39	49.84	79.79	91.10	97.98	$\bar{x}$
	300.7	1629.5	8244.5	26939.4	68674.5	88166.4	101156.6	Σx^2
	0.74	1.40	2.86	4.83	7.46	7.57	7.55	$S\bar{x}$
	1.68	3.10	6.40	10.90	16.86	17.12	17.10	$\pm$

Fig. II.2 The mean growth response of ten Fenugreek tissue cultures grown in the dark on MS medium with NAA 10 ppm and coconut water 10% v/v

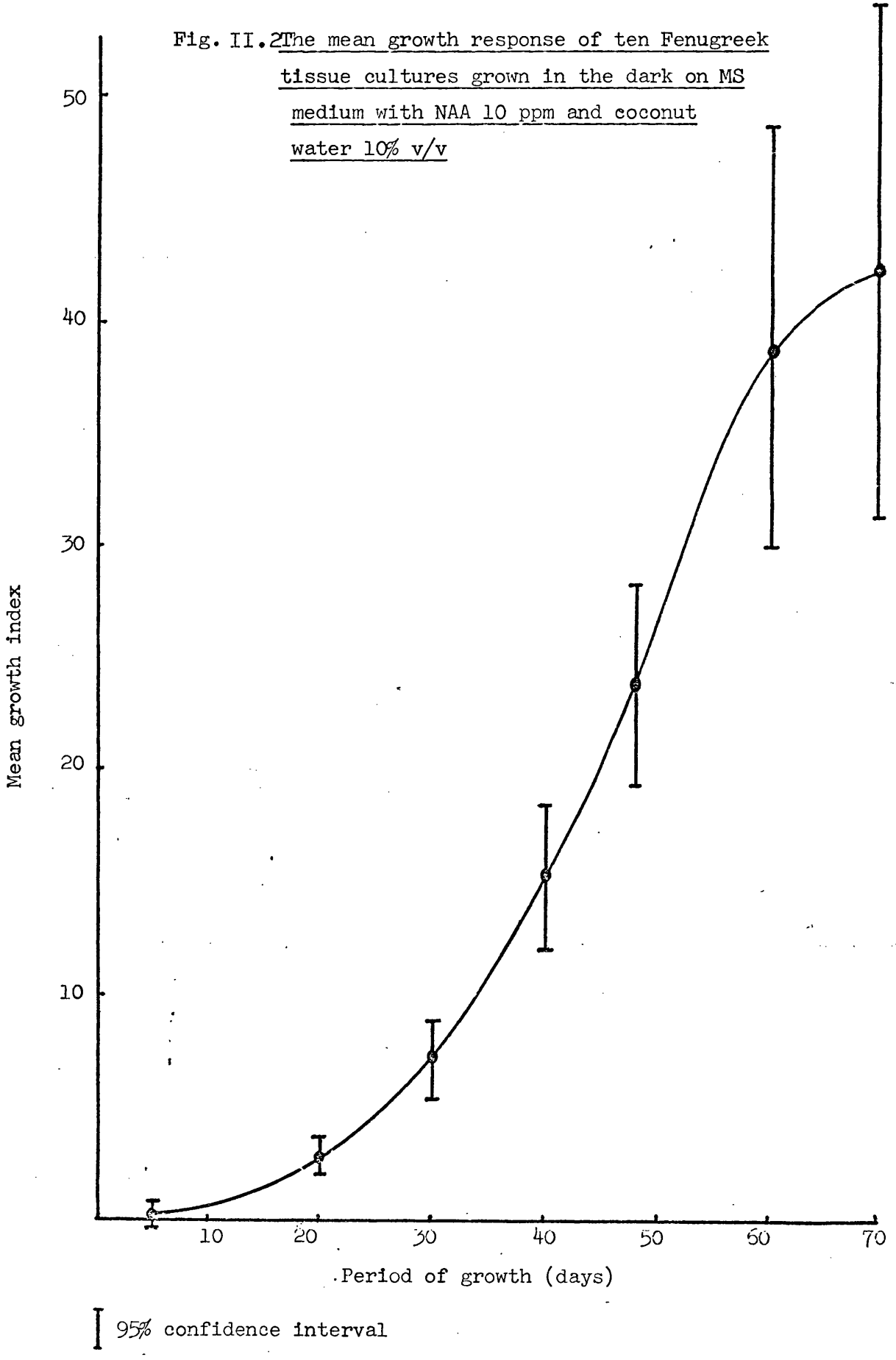
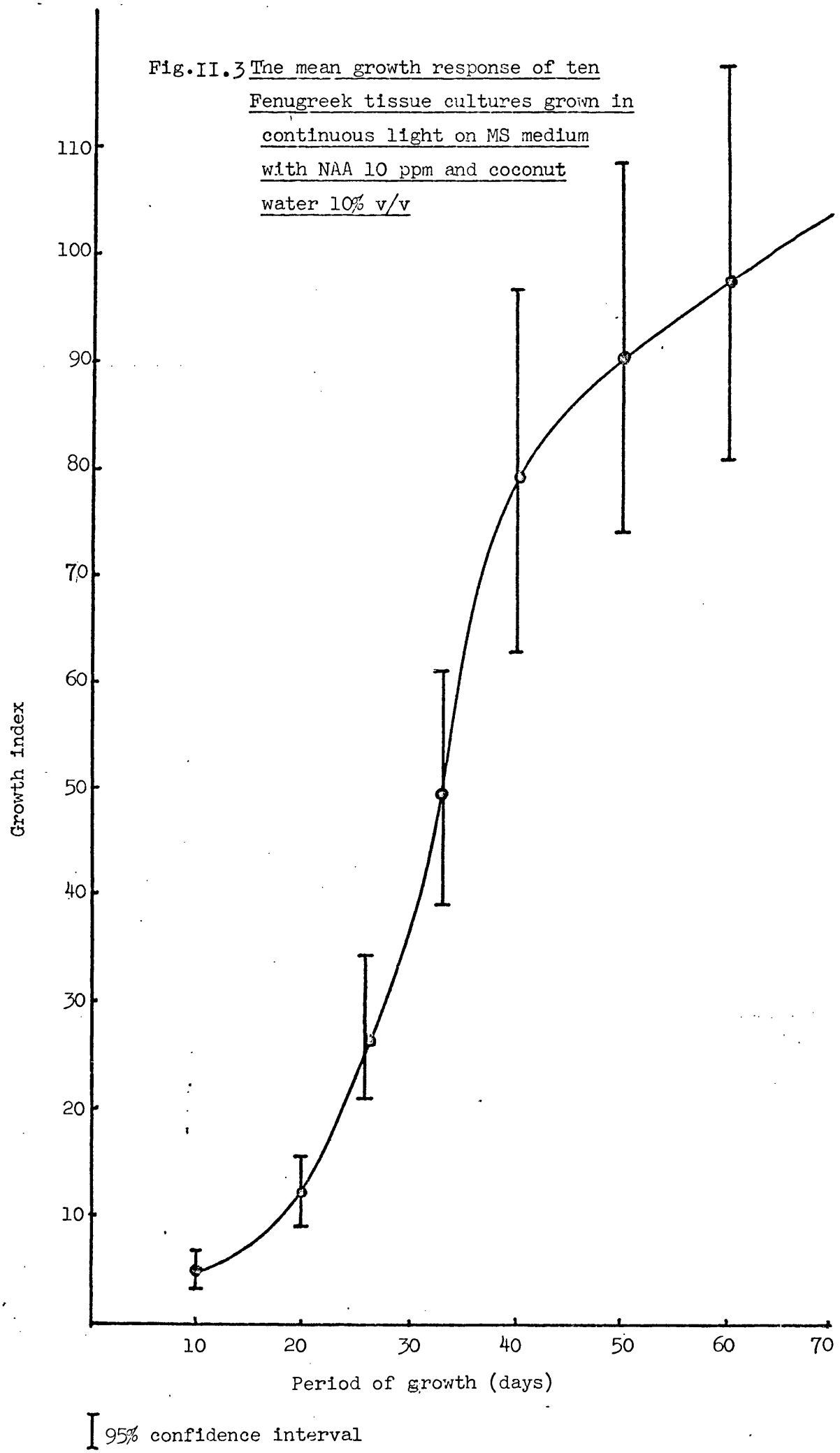


Fig.II.3 The mean growth response of ten Fenugreek tissue cultures grown in continuous light on MS medium with NAA 10 ppm and coconut water 10% v/v



days. A growth period of 35 days was chosen for subsequent experiments.

(2) Variation of NAA concentration in the induction medium

The auxin concentrations of the media used for induction was compared with media containing concentrations of .01, .1, 1 ppm and 20 ppm to see which gave the maximum increase in fresh weight in a 35 day growth period. Accurately weighed innocula of tissue were subcultured onto the trial media and grown in continuous light at 25°C for 35 days. Ten cultures were grown on each medium and at the end of the growth period each culture was harvested, weighed and the growth indices calculated, Table II.8

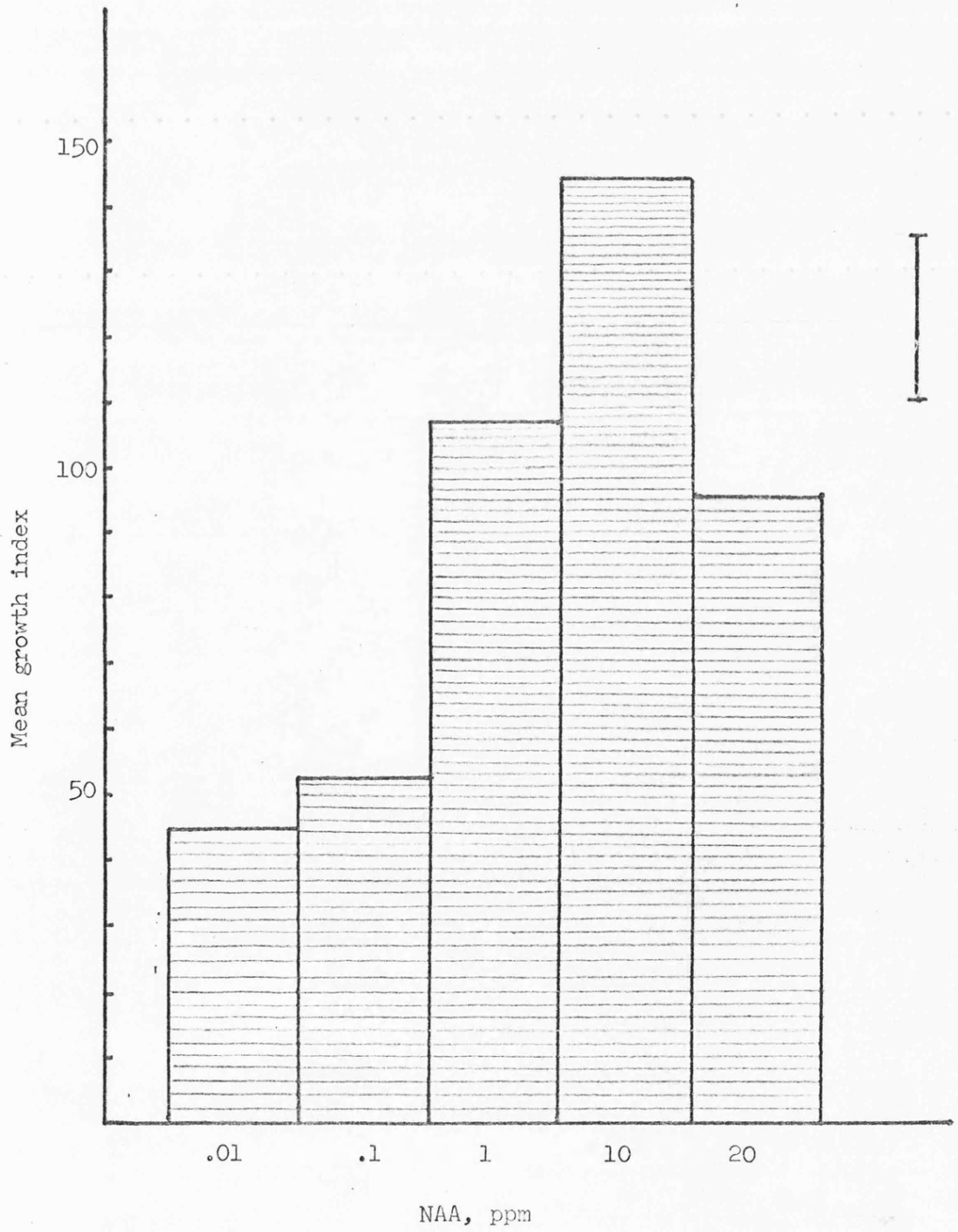
The results, Fig. II.4 show that 10 ppm NAA produced a significantly greater increase in fresh weight than the other concentrations of auxin. Tissues grown on 20 ppm were yellow and two had died after 35 days. Similarly .01 ppm gave poor unhealthy cultures, three of which had died by the end of the period. Concentrations of 0.1, 1.0 and 10 ppm all gave green healthy cultures. No organogenesis was observed in any of the cultures grown in this experiment. The NAA concentration of 10 ppm was maintained for stock cultures and a second line was initiated on 1 ppm NAA because the callus formed was more friable in texture, and might be more suitable for the initiation of suspension cultures. It was also hoped to compare the sapogenin and phytosterol constituents of the two cultures after sustained growth at different auxin/cytokinin ratios.

Table II.8

Growth indices of tissue cultures grown on MS medium with different concentrations of NAA and 10%v/v coconut water

.01 ppm NAA	.1 ppm NAA	1.0 ppm NAA	10 ppm NAA	20 ppm NAA
70.00	55.45	102.34	138.97	134.04
44.91	74.05	79.51	220.21	108.25
14.98	60.36	126.39	189.82	115.62
34.03	28.64	97.74	136.75	98.91
29.49	91.66	95.99	75.48	118.36
26.68	44.78	95.66	130.57	52.93
77.44	64.33	145.74	119.44	65.32
38.44	14.97	81.98	88.73	75.12
58.31	50.33	96.35	177.22	91.37
52.77	35.84	135.96	156.92	96.23
$\bar{x}$ 44.70	52.04	105.76	143.41	95.62

Fig.II.4 Growth response to different concentrations of NAA



(3) Variation of the volume of coconut water supplement in the induction medium

Coconut water is the liquid endosperm of the fruit of Cocos nucifera. When the embryo grows it sends out part of its cotyledons into the central cavity and forms a spongy cellular tissue which grows at the expense of the endosperm.

In 1941 J. van Overbeek[5] first used coconut water as a growth promoting supplement. Immature excised Datura embryos would only grow on a basal medium when it was supplemented with unautoclaved coconut milk. Caplin and Steward[29] found that tiny explants of secondary phloem from carrot root grew into an undifferentiated mass at many times the basal rate of growth if White's liquid medium was supplemented with 15% v/v of coconut water.

Many others have found that coconut water is a stimulant, which induces and maintains growth in a variety of plant cell cultures. Morel and Wetmore[30] obtained the first tissue culture from a monocotyledon by adding coconut water and 2,4-D to their medium. Tulecke[10] found that it promoted growth of a tissue culture obtained from Ginko pollen.

Steward and Shantz[12] attempted to isolate an active growth promoting factor from coconut milk. They succeeded in isolating 1,3-diphenylurea but it was only active in the stimulation of carrot cultures in the presence of casein hydrolysate. They finally concluded that the activity of coconut water results from the interaction of a number of substances.

Nitsch[31] broadly classified the constituents of coconut water as:

(i) An auxin which is unidentified. The activity of this compound is increased by autoclaving due to the hydrolytic release of more auxin from a bound state

(ii) Cytokinin in the form of zeatin riboside

(iii) A neutral fraction of the sugar alcohols sorbitol, scyllo inositol and myo inositol

(iv) A reduced nitrogen fraction

Tulecke[17] et al have carried out extensive studies on the chemical composition of coconut water from both mature and immature fruits. They reported the presence of shikimic and quinic acids which in combination with the other organic acids present, malic, citric, succinic and pyrrolidone carboxylic, were found to have growth promoting properties in holly stem cultures (Ilex aquifolium).

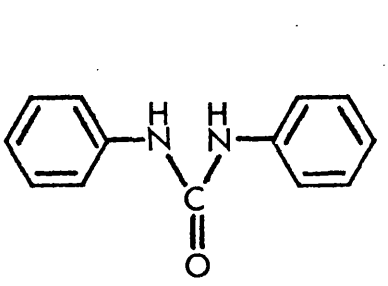
The commonly used concentration of 10% v/v was also found to provide a large sugar supplement of up to 1.4 g/litre. The sugars present were sucrose (890 mg), glucose (246 mg) and fructose (251 mg).

The relationship between the free amino acids and vitamins is thought to be a significant controlling factor in the growth of some plant tissue cultures. Paris, Duhamet and Goris[32] made up media containing the vitamins and amino acids which are known to occur in coconut water. Crown gall tissue grew as well on this medium as on medium containing whole coconut water. The use of amino acid or vitamins alone did not produce the same response.

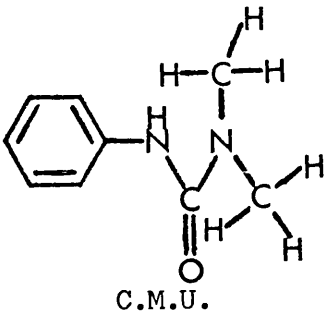
Tulecke[17] found a high content of free amino acids in the endosperm of mature fruits and calculated that 10% v/v

supplement would provide a medium concentration of 75.7 mg/ litre of total amino acid. He identified and determined the following amounts of the amino acids in the autoclaved water of mature fruits, Table II.9

Steward et al [12] have also isolated the free amino acids and none of the individual amino acids have shown any growth promoting activities although Steward [12] did show growth promotion by 1,3 diphenyl urea. This substance shows a striking chemical resemblance to the Dupont herbicide CMU.



1,3-Diphenyl urea



C.M.U.

The vitamins of coconut water have been studied by Vanderbilt [17] and those detected are shown in Table II.10

The mineral contents of coconut water were studied by McCance and Widdowson [17], Table II.11

Tulecke [17] observed that a 10% v/v supplement would only provide vitamins in sub-optimal concentrations for the growth of most plant tissues and that the growth of a specific tissue might be improved by enrichment with some of these substances.

An experiment was carried out to study the effects of different concentrations of coconut water supplement on the growth of Fenugreek tissue cultures. A series of media were prepared with supplements of 0, 5, 10, 15 and 20% v/v. No other source of cytokinin was added and the auxin concentration was maintained at 10 ppm NAA. Innocula were weighed and each

TableII.9

Amino acids present in autoclaved coconut water from mature fruit

Aspartic	11.4 µg/ml
Glutamic	104.9
Serine	85.0
Glycine	18.0
Asparagine	25.3
Threonine	27.4
Alanine	198.0
Glutamine	2.0
Histidine	TRACE
Lysine	13.0
Arginine	20.7
Proline	12.9
Valine	15.5
Leucine	33.0
Phenylalamine	TRACE
Tyrosine	TRACE
γ Aminobutyric	173.2
Hydroxyproline	8.2
Homoserine	8.8
Methionine	TRACE
Total	757.3

reproduced from ref.I7

Table II.IO

Vitamins present in coconut water

Compound	µg/ml
Nicotinic acid	0.64
Pantothenic acid	0.52
Biotin	0.02
Riboflavin	0.01
Folic acid	0.003
Thiamine	TRACE
Pyrodoxine	TRACE

reproduced from ref.I7

Table II.II

Minerals present in coconut milk

Mineral	Mg/100G
Potassium	312.00
Chloride	183.00
Sodium	105.00
Phosphorus	37.00
Magnesium	30.00
Sulphur	24.00
Iron	00.10
Copper	00.04

reproduced from ref.17

added to 20 ml of medium in plastic sterile jars. Murashige and Skoog [7] used pieces of callus weighing between 40 and 50 mg in their evaluation of media and similar weights were used in this experiment. The cultures were grown in continuous light (provided by warm white fluorescent tubes) for a period of 35 days, after which they were harvested and the growth indices calculated, Table II.I2

The results showed that the 10% v/v provided the maximum increase in fresh weight over the culture period. The yield was significantly greater than that of all but the 15% v/v trial medium, Fig. II.5 It was observed that the poorest growth occurred in cultures with no coconut water and hence no cytokinin and by the end of the culture period some of these cultures were showing signs of dying. Fenugreek tissue cultures would, therefore, appear to be dependent on the presence of exogenous cytokinin in the medium.

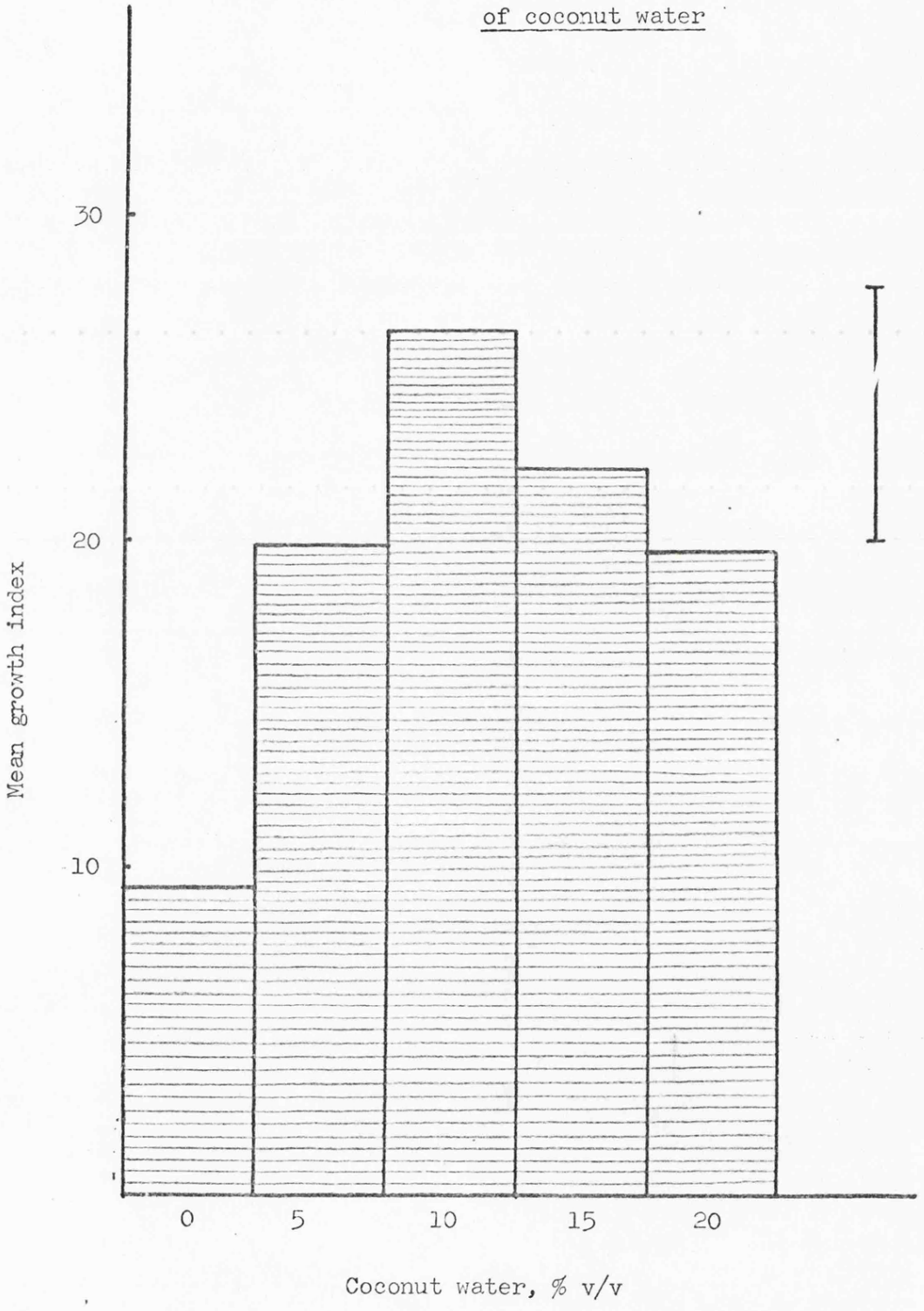
A supplement of 20% v/v gave a significantly lower yield than 10% v/v and it was concluded that some factor in the coconut water was inhibitory to growth at this concentration.

Table II.I2

Growth indices of tissue cultures grown on different concentrations of coconut water supplement

50 ml/l	100	150	200	0
16.60	16.60	20.87	12.7	7.47
25.74	38.94	19.27	19.39	10.58
24.54	34.89	20.17	25.87	7.76
22.89	32.19	27.99	33.23	11.54
16.21	16.60	29.90	26.37	13.73
16.99	16.76	15.08	12.94	4.69
18.25	29.37	21.44	13.60	7.63
10.06	15.89	24.92	18.60	6.49
18.96	33.18	18.52	16.60	9.78
26.66	28.31	23.77	15.43	9.71
19.69	26.27	22.19	19.47	8.938
196.9	262.7	221.9	194.73	89.38

Fig. II.5 Growth response to different concentrations of coconut water



(4) The use of 2,4-dichlorophenoxyacetic acid as a growth regulator

Of the growth regulators employed in tissue culture, 2,4-D has been widely used. Kaul and Staba[27] found that a concentration of 0.1 ppm provided the optimum concentration for diosgenin biosynthesis in Dioscorea deltoidea suspension and callus cultures. Heble et al[33], however, reported that diosgenin biosynthesis was enhanced in Solanum xanthocarpum tissues when 2,4-D was replaced by IAA or IBA, but the sitosterol content was reduced by three-fold. Tomita et al[34] cultured Dioscorea tokora cultures containing diosgenin on Linsmeier Skoog medium with a 2,4-D content of 10^{-6} M whilst Mehta and Staba[35] used 3 ppm 2,4-D for cultures of Dioscorea composita.

More recent work by Khanna, Mohan and Nag[36] reports the use of 1 ppm 2,4-D for tissue cultures of Fenugreek in an investigation of antimicrobials from plant tissue culture. Khanna and Jain[37] adopted the same concentration in their work on sapogenins from Fenugreek tissue and suspension cultures. Brain et al[38] employed a range of 0.1 to 1 ppm 2,4-D in suspension cultures of Fenugreek and obtained a maximum concentration of 0.081% m.f.b. of diosgenin with 0.1 ppm.

A concentration of 0.1 ppm was therefore found to be optimal for diosgenin biosynthesis in D. deltoidea and Fenugreek suspension cultures when used with Murashige and Skoog's medium. Khanna[37] found that 1 ppm gave tissue callus cultures with very high (1.8% m.f.b.) sapogenin levels, but these results have not been confirmed by other workers.

No data was available regarding the growth response of

Fenugreek tissue cultures to Murashige and Skoog's medium with 2,4-D as the auxin. An experiment was carried out to determine the optimum, and minimum inhibitory concentrations of 2,4-D with regard to increase in fresh weight. Trial media containing .01, 0.1, 1 and 10 ppm 2,4-D and 10% v/v coconut water, and free of NAA, were prepared and the growth response of the tissue compared with a 2,4-D free control medium, containing 10 ppm NAA and 10% v/v coconut water, Table.III.The conditions employed were as in the previous experiments.

There was no significant difference in the fresh weight increase on .01, 0.1, 1.0 ppm 2,4-D and 10 ppm NAA, Fig.II.6 The 10 ppm 2,4-D medium gave significantly less growth and it was concluded that this concentration was inhibitory. Increased concentrations of 2,4-D inhibited pigmentation and tissues of 1 ppm and 10 ppm were yellow. Tissues grown on .01 and .1 ppm were indistinguishable from that grown on 10 ppm NAA.

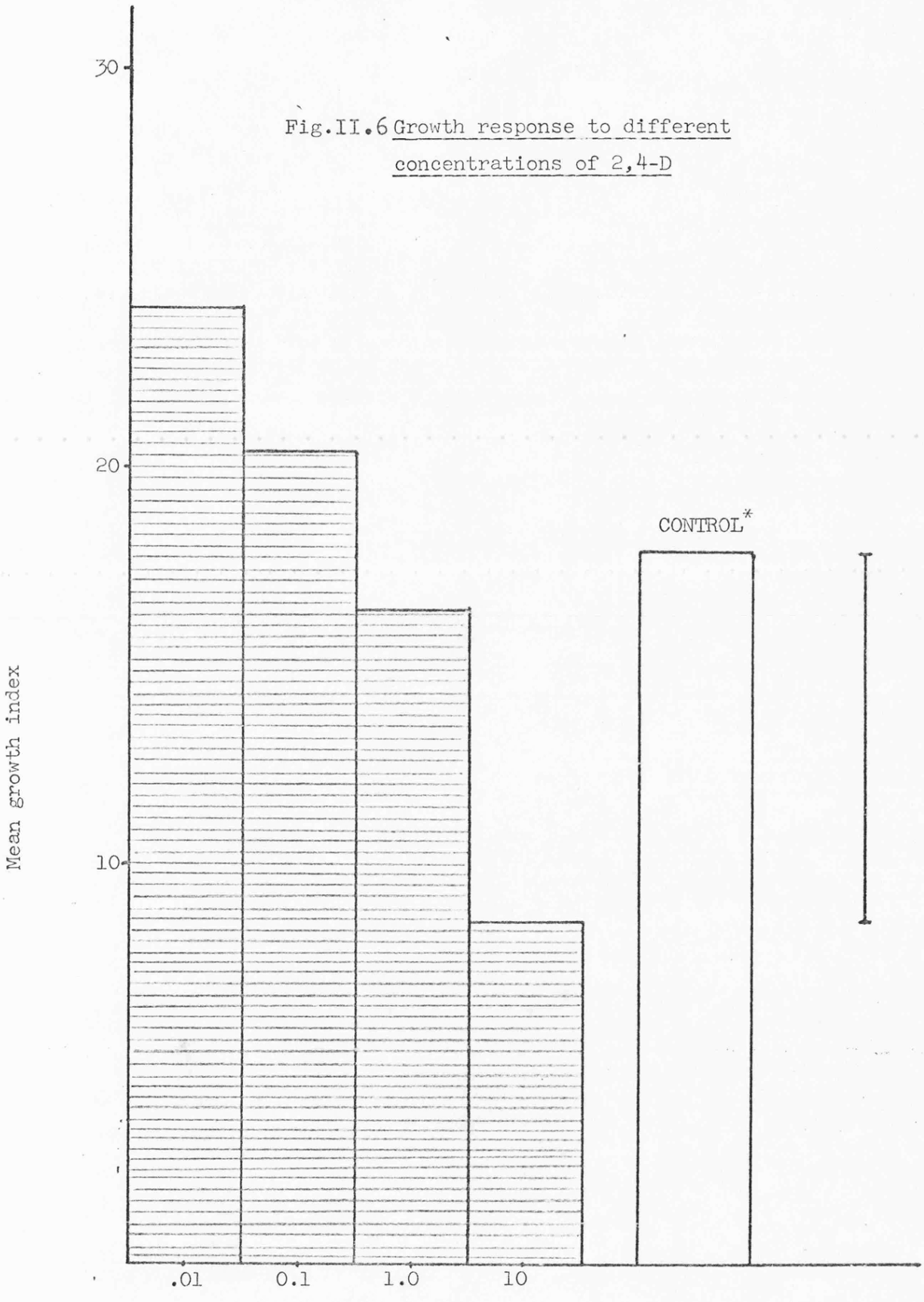
Cultures were subsequently maintained for two years on MS medium with 10% v/v coconut water and .01, 0.1 and 1.0 ppm 2,4-D.

Table II.3

The growth indices of tissue cultures grown on MS medium with different concentrations of 2,4-D

.01 2.4D	.1 2.4D	1.0 2.4D	10 ppm 2.4D	10 ppm NAA
24.0	26.2	17.7	9.4	13.7
30.0	22.9	14.7	2.8	8.4
16.0	17.9	14.6	6.1	20.4
23.7	19.8	11.7	14.6	22.5
23.6	21.6	11.2	15.2	9.7
27.5	22.4	8.0	4.6	36.9
27.0	15.8	9.7	16.8	13.0
14.8	13.9	28.6	5.2	13.3
30.5	21.7	16.5	3.6	17.11
22.7	22.9	31.6	6.2	24.5
$\bar{x}$ 24.0	20.50	16.43	8.45	17.95

Fig.II.6 Growth response to different concentrations of 2,4-D



(5) The growth response to NAA and kinetin

The use of coconut water as a supplement in the induction of tissue cultures is widespread and it is considered by Yeoman as difficult to surpass.[39] It provides a complex additive supplement anticipating the requirements of the proliferating tissue.

Because coconut water is subject to variation and its exact composition is not known, many workers subculture tissues onto defined medium after induction.

The tissues cultured in these experiments were initiated and maintained on the Murashige and Skoog[7] defined medium with a 10% v/v coconut water supplement. After six months the supplement was withdrawn from some tissues and replaced by an alternative source of exogenous cytokinin, in the form of kinetin. A series of media were produced in which the balance of kinetin and auxin varied as is shown in TableII.I4.

Weighed innocula were subcultured onto the media and the tissues were grown, as before, for a period of 35 days. The cultures were harvested, weighed and the growth indices calculated, TableII.I5

The results show a significantly greater increase in fresh weight of tissues grown with 10 ppm kinetin, at all auxin concentrations, than was achieved at any other concentration of kinetin, Fig. II.7 The control tissues grown on 10% coconut water and 10 ppm NAA were not significantly different from NKJ (.1 ppm NAA and 10 ppm kinetin) or NKL (10 ppm NAA and 10 ppm kinetin). Cultures grown on NKK (1 ppm NAA and 10 ppm kinetin) showed a significant difference from the control medium.

Table II.I4

Combinations of NAA and kinetin in trial media

NAA * / * Kinetin	.1	1	10
0	NK A	NK B	NK C
.1	NK D	NK E	NK F
1	NK G	NK H	NK I
10	NK J	NK K	NK L

* mg/litre

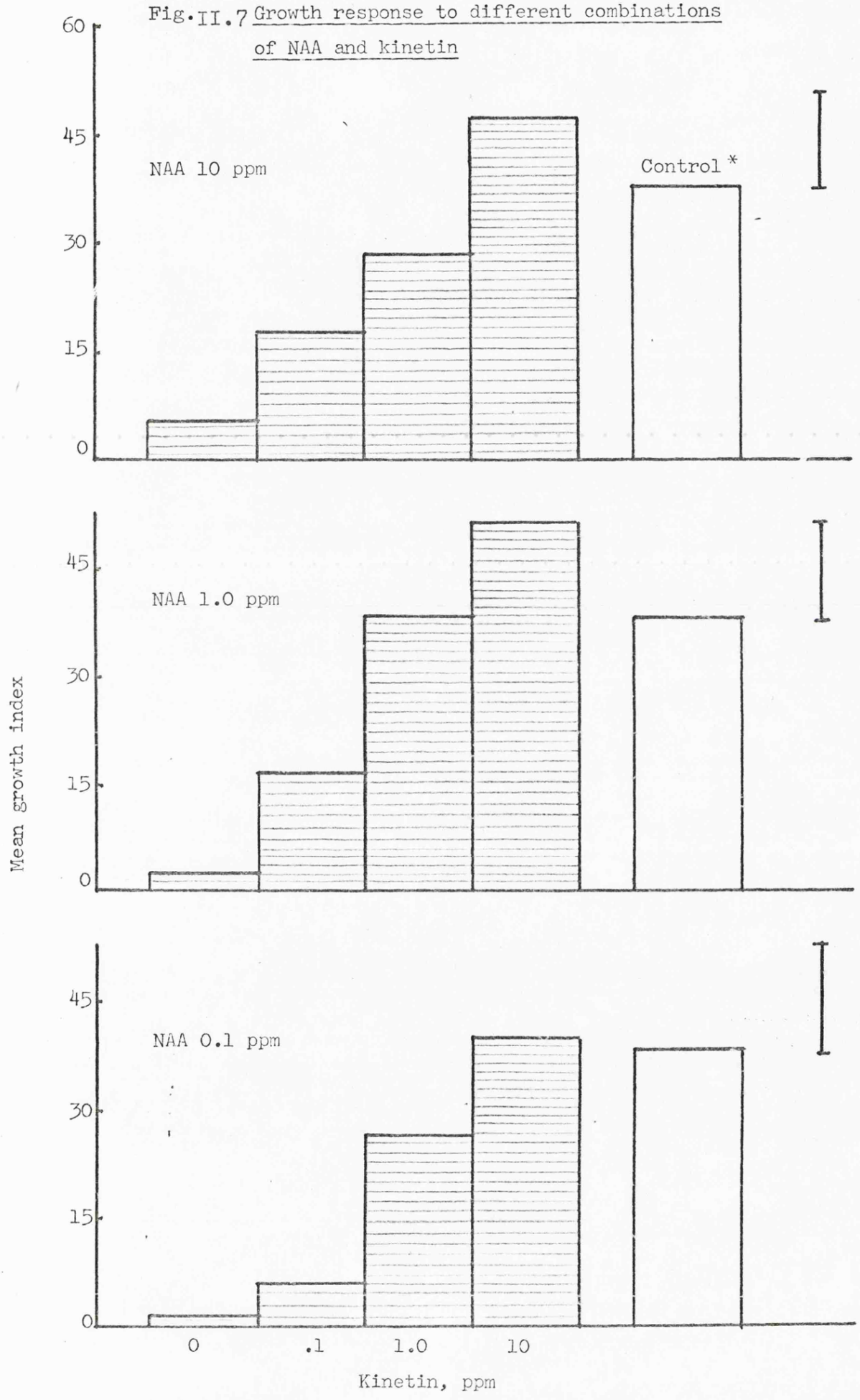
Table II.I5

Growth indices of tissue cultures grown on media containing combination of NAA and kinetin

NK MEDIA

A	B	C	D	E	F	G	H	I	J	K	L	Control
1.15	2.1	5.3	5.7	14.5	18.5	38.2	36.5	35.1	22.0	67.8	51.2	26.5
2.23	1.3	6.1	5.4	3.8	10.7	27.4	20.4	19.7	23.6	47.1	74.1	26.1
1.2	2.7	2.1	4.1	16.8	16.3	31.3	27.9	24.1	48.2	48.0	30.9	44.9
1.3	2.5	5.7	3.8	20.9	28.3	22.8	22.8	24.7	56.2	69.1	45.0	23.4
1.3	1.6	6.2	9.1	14.8	14.5	23.5	55.5	18.6	49.6	60.7	31.4	37.4
1.8	2.6	4.3	5.5	9.3	7.0	23.7	55.5	15.7	70.5	51.7	42.1	44.0
1.6	1.3	5.5	3.4	14.4	14.2	28.5	36.7	36.4	51.6	25.9	35.6	23.8
1.2	4.2	7.3	5.0	15.5	18.2	25.9	44.6	26.9	22.5	36.3	48.2	33.2
3.2	2.3	7.0	2.7	17.3	15.4	25.4	99.0	24.3	56.9	44.4	52.6	43.8
3.0	1.9	5.3	5.8	16.4	18.3	26.5	48.4	24.0	19.0	52.9	33.9	56.6
1.2	1.7	5.3	3.1	13.5	18.9	22.1	20.9	40.5	43.2	57.0	61.8	47.9
1.3	4.6	3.0	19.2	12.5	21.6	33.8	33.1	24.7	24.1	54.7	25.4	35.1
1.5	1.3	5.1	2.3	15.0	19.8	19.5	17.9	30.0	23.5	48.1	40.6	35.8
1.7	2.2	7.6	6.8	38.7	17.5	44.4	12.4	37.7	45.0	57.3	57.8	43.7
1.6	2.4	5.5	4.5	17.5	20.5	23.0	39.7	31.0	36.0	42.4	68.2	40.9
1.68	2.31	5.42	5.76	16.06	17.31	27.73	38.10	27.56	39.46	50.89	46.58	37.54

Fig.II.7 Growth response to different combinations of NAA and kinetin



* NAA 10 ppm, Coconut water 10% v/v

The removal of cytokinin from the media resulted in very little growth at all concentrations of NAA, confirming the work with coconut water that the tissue required exogenous cytokinin. In four replicates of NKA (.1 ppm NAA and 0 ppm kinetin) and three replicates of NKB (1 ppm NAA and 0 ppm kinetin) differentiation had taken place and primary root structures of approximately 2-4 mm in length were evident. No roots were observed on cultures grown on NKC (10 ppm NAA and 0 ppm kinetin). The tissues on NKC appeared healthier than those on NKA and NKB and resembled tissues grown in the presence of cytokinin in both colour and texture. Root formation is the most common form of organogenesis in both tissue callus cultures and suspension cultures.[40] The phenomenon was first observed by Nobécourt[40] in carrot cultures. and was subsequently reported by Skoog and Miller[41] and Torrey.[42] Roots will form from whatever part of the plant the explants are taken and are normally distributed irregularly over the surface of the callus. They resemble normal roots in the meristem and differentiated area behind, but the base ends blind in the parenchyma.

Gautheret[43] (1945) first showed that auxin at optimal concentrations could induce root primordia. The classical example, demonstrated by Miller and Skoog[41], was on pith parenchyma of tobacco callus. In this case high auxin and low kinetin levels induced root formation whilst a high kinetin to auxin ratio resulted in the initiation of shoots. Reinert[40] states that there are few observations with other tissue cultures supporting the principle that organogenesis is regulated by quantitative shifts in the ratio of hormones. Stichel[40] (1959) concluded that in many of the cases of

reported root formation the absolute concentration of the auxin in the medium is a controlling factor. Little is known about the influence of exogenous factors on organogenesis. Low light intensity has been shown to stimulate bud formation in tobacco cultures whilst high light intensity will inhibit it.[44] It has also been demonstrated that light can exert a positive influence on rhizogenesis in artichoke cultures.[45]

It would not appear from the results of this experiment that root induction in Fenugreek cultures is solely dependant on the absolute concentration of auxin. No roots were observed in any of the callus grown on media containing even as little as 0.1 ppm kinetin. The absence of root formation at the highest auxin level could possibly be explained by the fact that in carrot and pea cultures auxin has proved inhibitory at certain concentrations.[40] Kinetin or high auxin concentrations therefore appear to be inhibitory to root formation but no definite conclusion can be drawn because the influence of physical factors, such as light and temperature, have not been studied.

None of the media at the highest auxin and kinetin concentrations in this trial demonstrated an inhibitory effect on the growth of the cultures and a second experiment was carried out with a range of kinetin concentrations up to a maximum of 20 ppm, Table II.16 The largest concentration used with this medium in the growth of tobacco callus cultures by Murashige and Skoog was 10 ppm.

(6) Further investigation of the growth response to NAA and and kinetin

The results of the second trial are given in Table II.I7 A concentration of 20 ppm of kinetin did not produce a significant difference in the fresh weight increase from 10 ppm kinetin at the NAA concentrations used, Fig.II.8.At all kinetin concentrations the media containing 0.1 ppm NAA gave lower fresh weight increases than the media containing 1 and 10 ppm NAA. Only one medium NKBH produced a greater increase than the control.

It was concluded that the tissue requires an exogenous source of cytokinin and growth can be sustained on the MS medium in the absence of coconut water. The increase of kinetin concentration above 10 ppm gave no further increase in fresh weight yield under the conditions employed in the two experiments.

In both experiments only the medium containing 10 ppm kinetin and 1 ppm NAA showed a significant increase in yield over the control medium containing 10 ppm NAA and 10% v/v coconut water.

Table II.16

Combinations of NAA and kinetin in media used in the second trial

NAA * / Kinetin *	.1	1	10
1	NKB A	NKB B	NKB C
5	NKB D	NKB E	NKB F
10	NKB G	NKB H	NKB I
20	NKB J	NKB K	NKB L

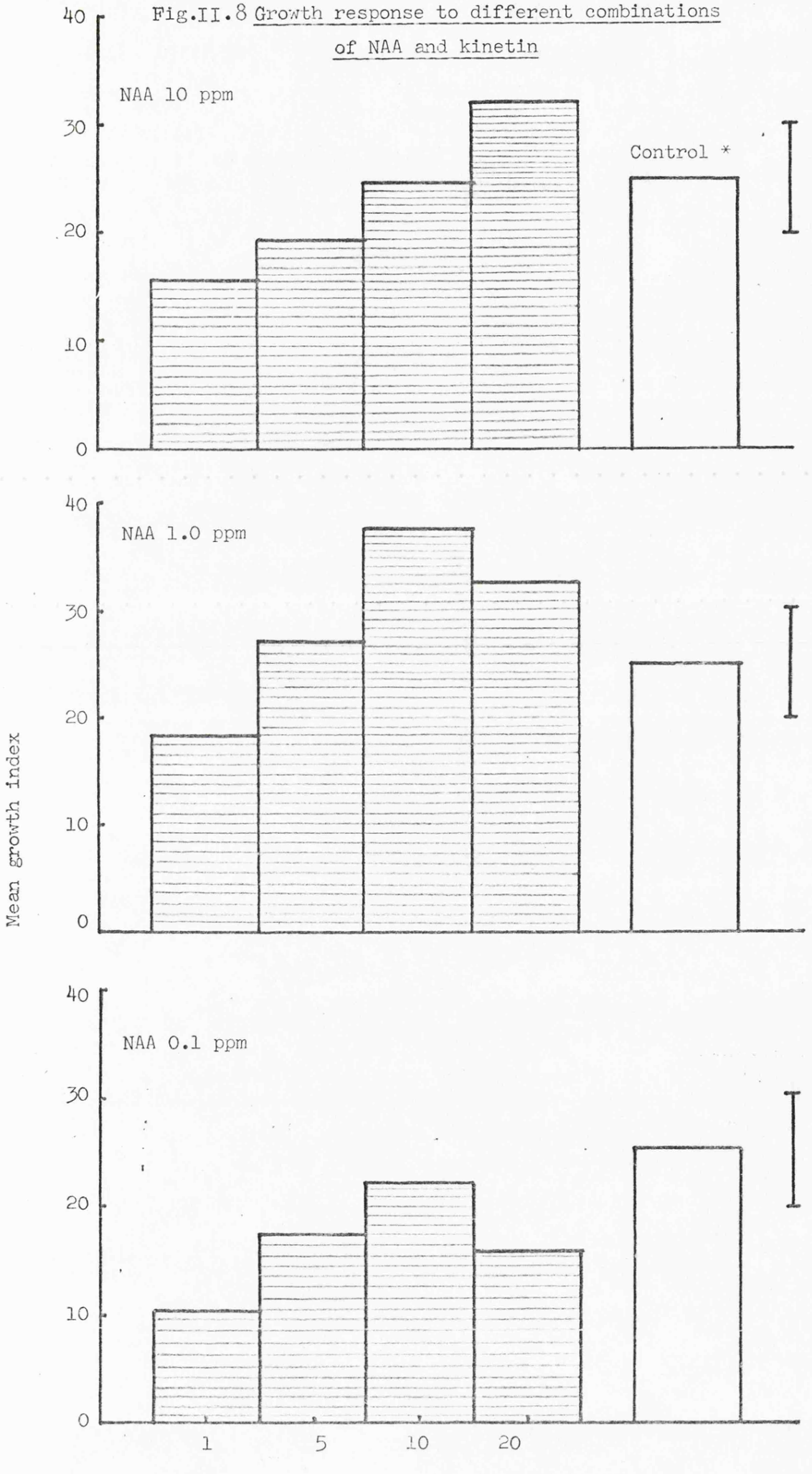
* mg/litre

Table II.I7

Growth indices of tissue cultures grown on media containing combinations of NAA and Kinetin

CON NKB	NKB A	B	C	D	E	F	G	H	I	J	K	L
24.89	11.75	15.11	22.11	20.58	43.98	20.19	26.43	36.32	32.03	17.68	21.68	25.68
29.69	17.84	19.11	28.77	19.44	39.60	21.31	26.65	27.15	13.11	35.64	33.13	27.10
18.67	11.70	28.14	24.78	20.93	21.55	24.82	35.59	52.68	32.19	22.67	43.05	31.75
21.81	9.06	20.00	20.17	22.91	10.19	15.88	30.06	23.96	25.28	8.88	45.34	37.37
23.92	11.25	7.75	11.31	9.29	33.76	18.90	9.27	54.10	24.25	21.18	38.53	56.56
23.89	14.74	6.29	12.91	17.27	32.73	19.89	26.14	34.82	28.34	17.33	46.10	27.10
29.56	4.26	24.39	15.75	12.47	22.17	25.88	18.24	58.47	12.18	21.55	48.85	32.68
41.55	12.48	12.73	10.62	16.27	31.02	20.88	15.62	30.47	21.91	31.57	20.34	25.91
24.36	7.24	14.05	11.13	13.92	16.61	19.70	22.04	25.74	24.20	18.77	48.32	40.51
25.74	12.37	23.69	14.36	15.32	31.47	20.66	13.13	53.36	47.31	19.64	20.81	35.79
37.24	11.65	27.07	16.81	18.10	26.91	14.44	20.03	27.35	12.57	11.28	25.61	31.51
17.95	5.79	12.27	10.36	12.10	27.03	13.98	24.58	23.27	29.77	9.61	36.46	27.05
20.08	8.79	27.33	14.84	21.68	33.86	16.03	28.77	65.69	20.48	10.97	20.79	31.25
17.45	10.26	11.94	13.96	19.43	21.90	16.48	17.68	25.10	21.68	18.90	19.92	19.92
16.12	4.9	27.07	6.33	17.36	12.50	20.08	13.76	25.98	20.55	10.10	20.87	29.57
24.86	10.26	18.46	15.61	17.13	27.02	19.27	21.80	37.63	24.39	18.39	32.65	31.98

Fig.II.8 Growth response to different combinations of NAA and kinetin



* NAA 10 ppm, Coconut water 10% v/v

(7) Growth response to 2,4-D and kinetin

A similar experiment was carried out with tissues growing on MS medium with 2,4-D. In a previous experiment concentrations of .01, .1 and 1 mg/litre had maintained the growth of tissues on MS medium with coconut water supplement.

After six months growth the supplement was withdrawn from sub-cultures of the tissue grown on .01 ppm 2,4-D. A series, of media, containing 2,4-D and kinetin, were prepared as shown in the table and cultures were grown and evaluated as before. (Table II.I8) Cultures grown on .01 ppm 2,4-D gave increased yields of tissue with increased kinetin, but the maximum yield, at 10 ppm kinetin, was not significantly better than the control, Fig. II.9 At higher concentrations of 2,4-D, changing the amount of kinetin in the media had little effect on the growth.

Table II.18

Combinations of 2,4-D and Kinetin in trial media

2,4-D * / * Kinetin	.01	.1	1
0	DK A	DK B	DK C
.01	DK D	DK E	DK F
.1	DK G	DK H	DK I
1	DK J	DK K	DK L
10	DK M	DK N	DK O

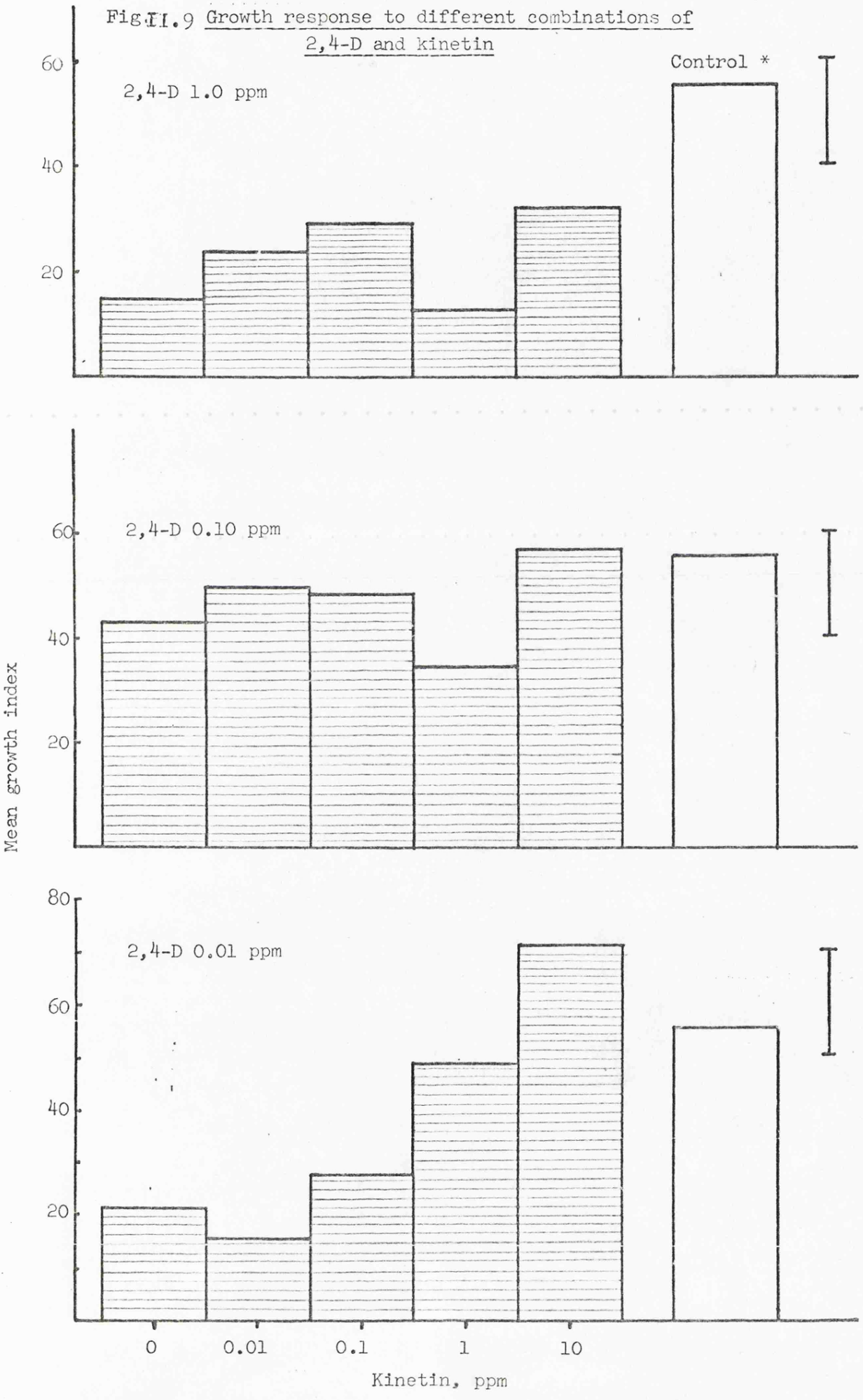
* mg/litre

Table II.I9

Growth indices for tissue cultures grown on media containing combinations of 2,4-D and kinetin

A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	CONTROL
25.29	27.05	13.71	26.16	62.92	15.95	16.78	47.53	13.69	45.22	19.46	20.01	77.13	77.48	30.00	43.29
29.55	37.61	18.69	21.19	40.70	33.73	23.46	50.54	30.26	32.16	8.69	7.72	41.73	49.63	50.37	115.27
32.83	44.33	10.63	14.81	47.16	27.33	15.70	38.16	14.20	36.17	38.91	5.19	42.17	52.64	20.83	68.99
22.46	27.52	21.10	21.37	24.57	36.71	21.43	50.94	32.64	22.42	24.39	1.57	101.86	31.48	19.31	93.88
19.58	66.66	10.46	17.82	71.59	22.58	47.90	44.15	35.31	28.34	24.92	42.59	84.35	35.14	7.32	44.79
23.04	70.49	11.45	11.92	39.30	12.39	73.32	43.23	19.01	40.96	51.26	4.76	95.71	57.14	28.91	37.09
20.63	34.79	19.10	19.23	39.05	11.59	26.48	66.66	34.19	60.72	14.36	17.83	84.44	64.46	15.29	36.28
17.83	69.88	19.21	9.75	46.73	18.43	27.24	62.19	19.36	53.30	52.93	12.36	52.24	55.00	46.91	23.88
23.67	42.91	14.80	16.73	52.95	37.40	25.03	76.38	24.71	56.92	25.70	9.37	62.09	60.73	21.07	76.96
21.42	41.25	15.88	19.36	31.00	24.15	28.44	30.51	48.02	49.99	23.52	14.33	48.73	66.61	41.5	33.58
33.05	49.46	16.64	11.83	42.62	25.17	28.23	37.61	28.19	23.05	50.21	22.55	72.73	77.17	28.10	67.78
9.77	38.05	2.42	10.55	58.75	21.68	8.22	42.35	28.39	45.79	19.94	7.53	34.64	66.47	44.51	55.80
9.21	27.72	9.10	10.43	76.17	18.44	15.12	47.35	28.41	39.87	51.83	5.63	70.52	39.21	22.00	41.85
17.67	33.45	23.76	15.65	52.34	32.59	24.19	34.74	24.03	39.05	79.61	6.50	124.87	67.29	36.80	43.21
32.72	32.65	12.60	11.29	57.10	16.78	28.01	47.05	52.88	48.88	32.81	11.17	67.61	50.77	58.20	41.59
22.50	42.90	14.63	15.87	49.52	23.66	27.30	47.99	28.88	41.52	34.57	12.61	70.72	56.74	31.47	54.95

Fig.II.9 Growth response to different combinations of 2,4-D and kinetin



* NAA 10 ppm. Coconut water 10% v/v

THE ADDITION OF MEVALONIC ACID TO THE CULTURE MEDIUM

The incorporation of mevalonic acid (MVA) into the biosynthetic pathways of the plant growth regulators has been demonstrated. In Gibberella fujikoroi[46] MVA was shown to be incorporated into the synthesis of gibberellins via a diterpene precursor geranyl geranyl pyrophosphate. Tritium labelled mevalonate was utilised in the synthesis of abscisic acid in avocado pears.[47] Chen and Hall[48] demonstrated that the incorporation of labelled MVA into tobacco pith cultures partially replaced the culture requirements for cytokinin. The 2-C^{14}-mevalonic acid was specifically incorporated into the N^6-(Δ^2-isopentenyl)adenosine residues of t-RNA and it was postulated that MVA was a precursor of the natural cytokinins. Subsequently, McChesney[49] obtained growth stimulation in some tissue cultures when a supplement of MVA was added to the culture medium containing the auxin and cytokinin normally required by the tissue. Furthermore, the optimum concentration of MVA for a growth stimulation was very low (10^{-7}M) and it was considered that MVA was acting as a hormone, rather than a nutrient. McChesney[49] described the type of growth stimulation as being different to that obtained with gibberellins and concurred with the hypothesis of Chen and Hall[48] that MVA was converted to a natural cytokinin in some of the cultures examined. The hypothesis was supported by his success in establishing a cytokinin independent, MVA dependent, tissue culture from a normally cytokinin-dependent culture. McChesney[49] recommended the routine incorporation of an MVA supplement into the media of slow growing cultures where the usual synthetic cytokinin agents

did not prove satisfactory.

An experiment was performed in which MVA was added to the MS medium, containing 10 ppm NAA and 10% v/v coconut water, on which Fenugreek tissue cultures had been established. The range of concentrations employed by McChesney was adopted and the increase in fresh weight of cultures was recorded after a 35 day growth period. MVA was added to autoclaved, cooled, medium, after sterilisation by membrane filtration, to provide final concentrations of 10^{-3}, 10^{-5}, 10^{-7}, 10^{-9} and 10^{-11} moles/litre. Tissues of known weight were grown in sterile plastic jars containing 20 ml of medium at $25^{\circ} \pm 1^{\circ}$ in continuous light (1500-1800 lux). Fifteen replicates were grown on each of the trial media and on a control medium containing no MVA supplement.

McChesney [49] examined nine different cultures for MVA growth stimulation, but only obtained a response from five. The growth rates of three cultures were unaffected and with one culture he observed a slight inhibition of growth. Four of the five stimulated cultures were strains of tobacco callus, and cultures of different plant species, such as soybean and H. gracilis, were not stimulated. In the experiment with Fenugreek cultures, no difference in growth response between the trial and control cultures was observed, (Table II.20). It was concluded that the medium may itself have provided the optimum conditions for Fenugreek callus growth and that a supplement of MVA was unnecessary or, one or more constituents of the medium may have been a limiting factor. It is also possible that the effect observed by McChesney is limited to certain callus cultures, particularly those of tobacco. Mevalonic acid was not included in the medium formulation for Fenugreek cultures.

Table II.20

The growth indices of tissues grown on MS medium with Mevalonic acid supplements

MVA Moles/litre					
0	10^{-11}	10^{-9}	10^{-7}	10^{-5}	10^{-3}
26.4	48.8	27.2	35.2	23.1	14.4
22.7	16.8	35.2	36.2	20.4	13.3
15.5	14.3	17.3	18.9	21.6	17.4
41.0	34.0	19.9	24.6	53.8	25.5
35.9	34.0	23.2	34.1	34.6	44.5
39.7	32.6	23.1	43.2	30.8	29.4
23.3	46.5	21.2	26.9	29.1	39.3
22.2	13.2	24.2	21.0	15.4	32.7
27.9	29.4	12.9	13.9	31.0	28.6
33.5	19.5	10.5	23.1	27.0	26.6
13.0	12.2	23.6	45.7	20.7	23.2
29.3	23.6	18.1	23.7	25.0	18.3
31.8	22.7	24.5	52.5	23.9	23.7
34.1	19.4	35.8	21.4	18.4	23.8
27.5	25.8	27.1	29.6	19.1	26.9
Mean 28.3	26.1	22.9	30.0	26.3	25.6

PART II CHAPTER III

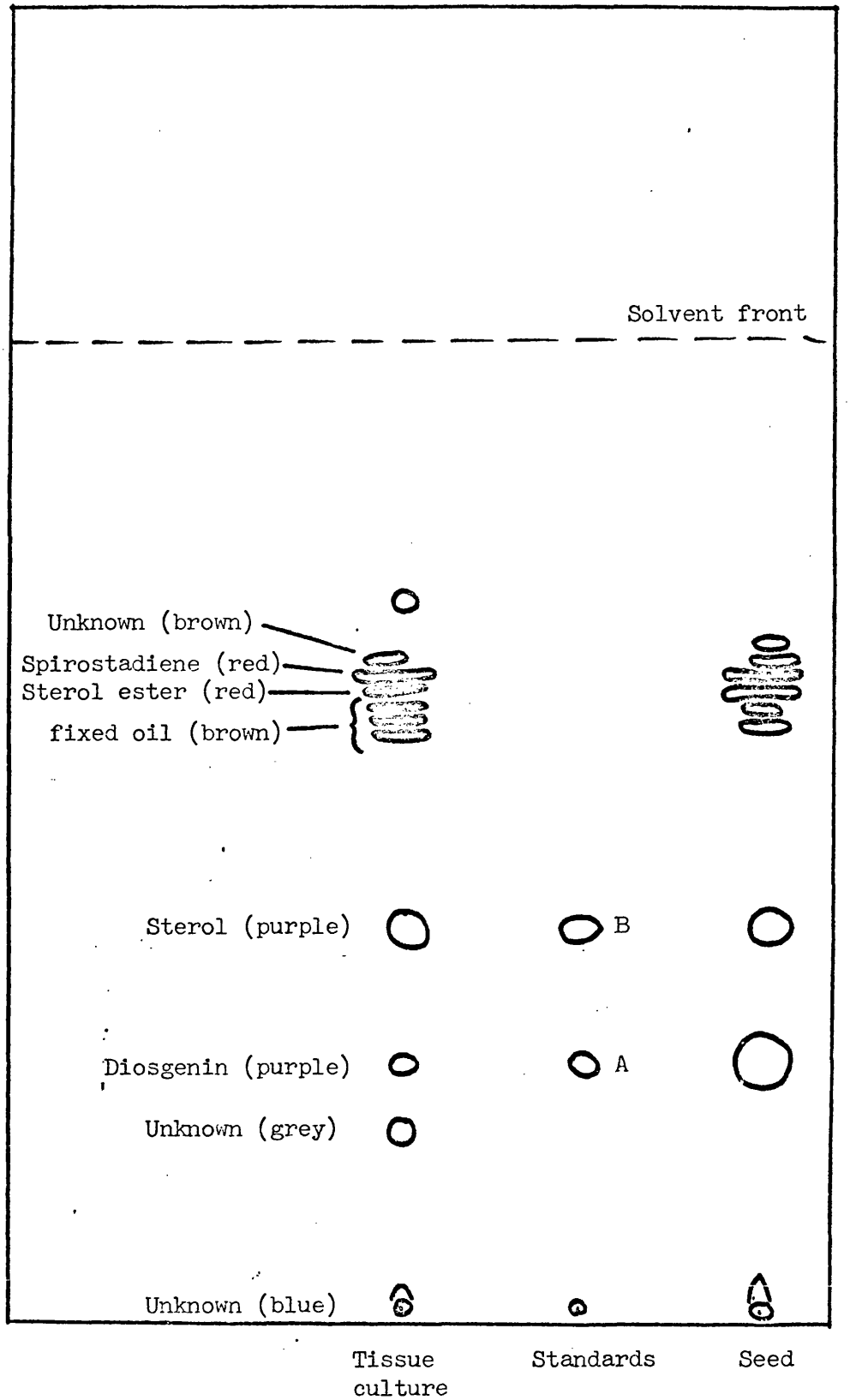
THE IDENTIFICATION OF PHYTOSTEROLS AND SAPOGENINS IN ONE YEAR OLD TISSUE CULTURES

THE EXTRACTION AND EXAMINATION BY T.L.C. OF PHYTOSTEROL AND SAPOGENIN FRACTIONS

Spirostanols and phytosterols were isolated from a one year old tissue culture which had been repeatedly sub-cultured at 30 day intervals. A sample of callus grown in continuous light, on MS medium with 10 ppm NAA and 10% v/v coconut water, was dried and hydrolysed with 2N hydrochloric acid. The residue was made alkaline, dried and extracted for 24 hours with light petroleum. The crude extract obtained contained the free and aglycone phytosterol and sapogenin fractions.

Thin layer chromatography of the extract on a silica gel G layer, with a solvent of hexane:ethyl acetate 4:1, gave spots corresponding in colour reaction and Rf value to spots of standard diosgenin 0.27 and sitosterol 0.4. A crude, hydrolysed petrol soluble extract, obtained from 2.5 g of the seed RH.2336, from which the tissue cultures were derived, was also run for comparison. The plates were sprayed with antimony trichloride (30%% in conc. hydrochloric acid) and the distinctive purple colour, given by diosgenin and phytosterol on warming, occurred in both the standard and unknown spots. The spots obtained were similar in both extracts, Fig. II.10 In the seed extract the intensity of the spots corresponding to diosgenin and sitosterol standards were of similar intensity. In the tissue culture

Fig. II.IO TLC examination of crude steroid extracts from Fenugreek tissue cultures and seed.



A Diosgenin

B -sitosterol

extract the spot corresponding to diosgenin was weaker than the spot corresponding to sitosterol. The compounds corresponding to diosgenin and sitosterol were isolated by preparative T.L.C. (see experimental) from callus extract. Sufficient material, approximately 800 µg of phytosterol and 200 µg of sapogenin, was collected for a G.L.C. analysis.

THE IDENTIFICATION OF PHYTOSTEROLS AND SAPOGENINS

(1) Analysis of phytosterols by G.L.C.

Gas liquid chromatography has been used by many workers in the examination of phytosterols.[34 50 51] The use of OV101[34 52] or SE30 columns is common, with either nitrogen, or helium, carrier gas. Grunwald[53] preferred to use SE30 and helium carrier gas for the analysis of free phytosterols, because he obtained a better separation and less tailing, without the necessity of forming T.M.Si-ethers. With nitrogen carrier gas and OV101 stationary phase columns, Jefferies[52] was unable to obtain the same retention times, or weight relative sensitivities as Grunwald, and found it necessary to form T.M.Si derivatives and reduce the length of the columns from 2 to 1 metre.

The 1 metre OV101 columns did not provide a satisfactory separation of the phytosterol extract of callus, because of the presence of a second major peak adjacent to sitosterol, Fig.II.12.

A pair of 6 ft stainless steel columns, packed with SE30 2½% on chromosorb G 80-100 mesh, gave a satisfactory separation with sterol T.M.Si-ethers, at their maximum working temperature of 250°, with nitrogen carrier gas. Geuns[51] had used 2 metre OV-17 3% columns for sterol analysis and a pair were packed and compared with the

SE30 columns. The OV-17 columns gave no better separation than the SE30's and required a higher working temperature of 270°C for the same retention times. The final conditions chosen for the initial analysis of the sapogenin and phytosterol fractions were; SE30 2½% 6 ft ¼" columns, with injection temperature of 280°C, oven temperature of 250°C and nitrogen carrier gas at 60 ml/min. All fractions were introduced as T.M.Si-ethers.

(2) The determination of phytosterol retention times relative to cholestane

The mixed phytosterol T.M.Si-ether was prepared with an internal standard of cholestane, and the retention times of unknown peaks relative to the internal standard, compared with similarly treated pure sterol standards, Table II.2I

Four peaks were obtained, three of which corresponded to the standards of cholesterol, campesterol and sitosterol, previously reported in Fenugreek seed, Fig.II.1I It was thought that the major peak adjacent to the sitosterol was either diosgenin impurity, or stigmasterol. The relative retention time corresponded exactly with stigmasterol standard and further investigation by T.L.C. showed no sapogenin in the phytosterol extract. The proximity of the stigmasterol and sitosterol peaks was the reason why the 1 metre OV101 columns proved unsatisfactory for the callus extracts (see Fig.II.I2). Jefferies[52] had found that 90% of the phytosterol from the seed was sitosterol and his separations were not troubled by stigmasterol.

Table II.2I

Relative retention times of standards and unknowns T.M.Si-ethers with cholestane as an internal standard

Mean of two determinations.

Standards	
Cholesterol	2.10
Campesterol	2.55
Stigmasterol	2.76
Sitosterol	3.16
Diosgenin/yamogenin	2.83

Phytosterol fraction	
A	2.10
B	2.60
C	2.80
D	3.30
E	2.36

Sapogenin fraction	
	2.82

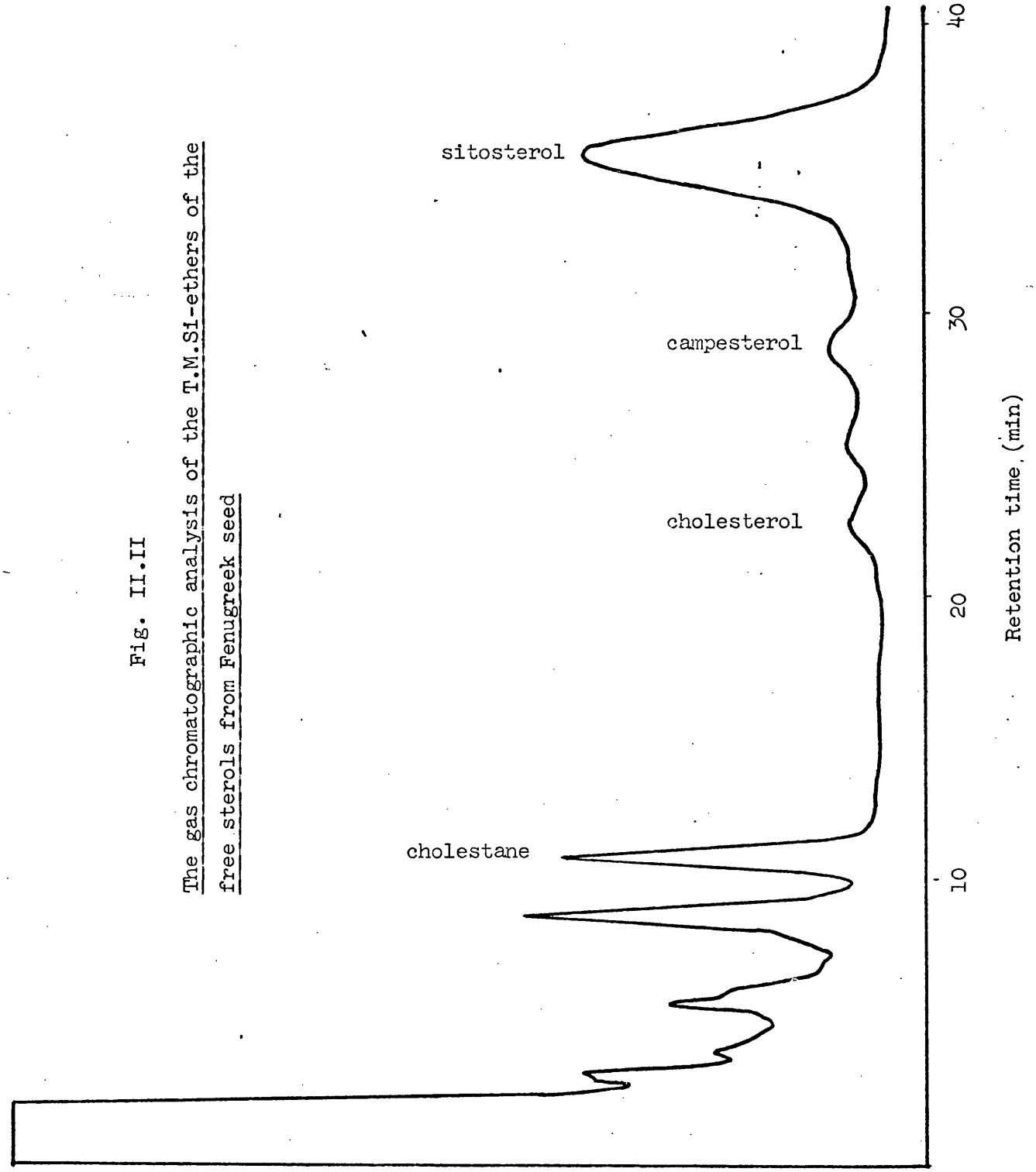
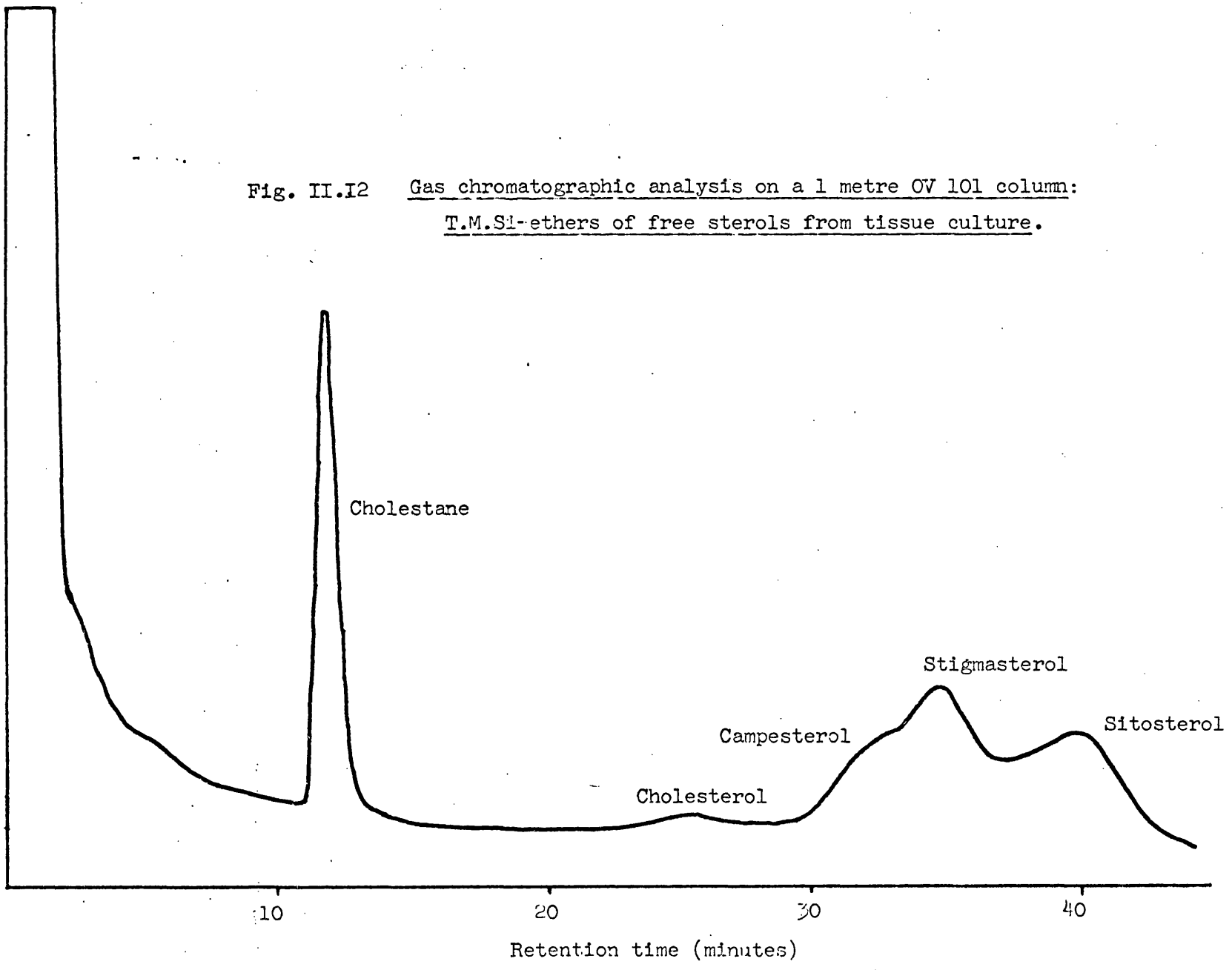


Fig. II.II

The gas chromatographic analysis of the T.M.Si-ethers of the free sterols from Fenugreek seed

Fig. II.I2 Gas chromatographic analysis on a 1 metre OV 101 column: T.M.Si-ethers of free sterols from tissue culture.



(3) G.L.C. analysis of the sapogenin fraction

The sapogenin fraction, corresponding to the diosgenin standard, was analysed as a T.M.Si-ether, as before. The sample gave one peak, Fig.II.13, and the relative retention time of this corresponded to that of a diosgenin/yamogenin standard mixture.

It was found that a T.M.Si-ether of the standard diosgenin/yamogenin mixture gave sharper peaks, with greater sensitivity and less 'tailing', than the free sapogenin.

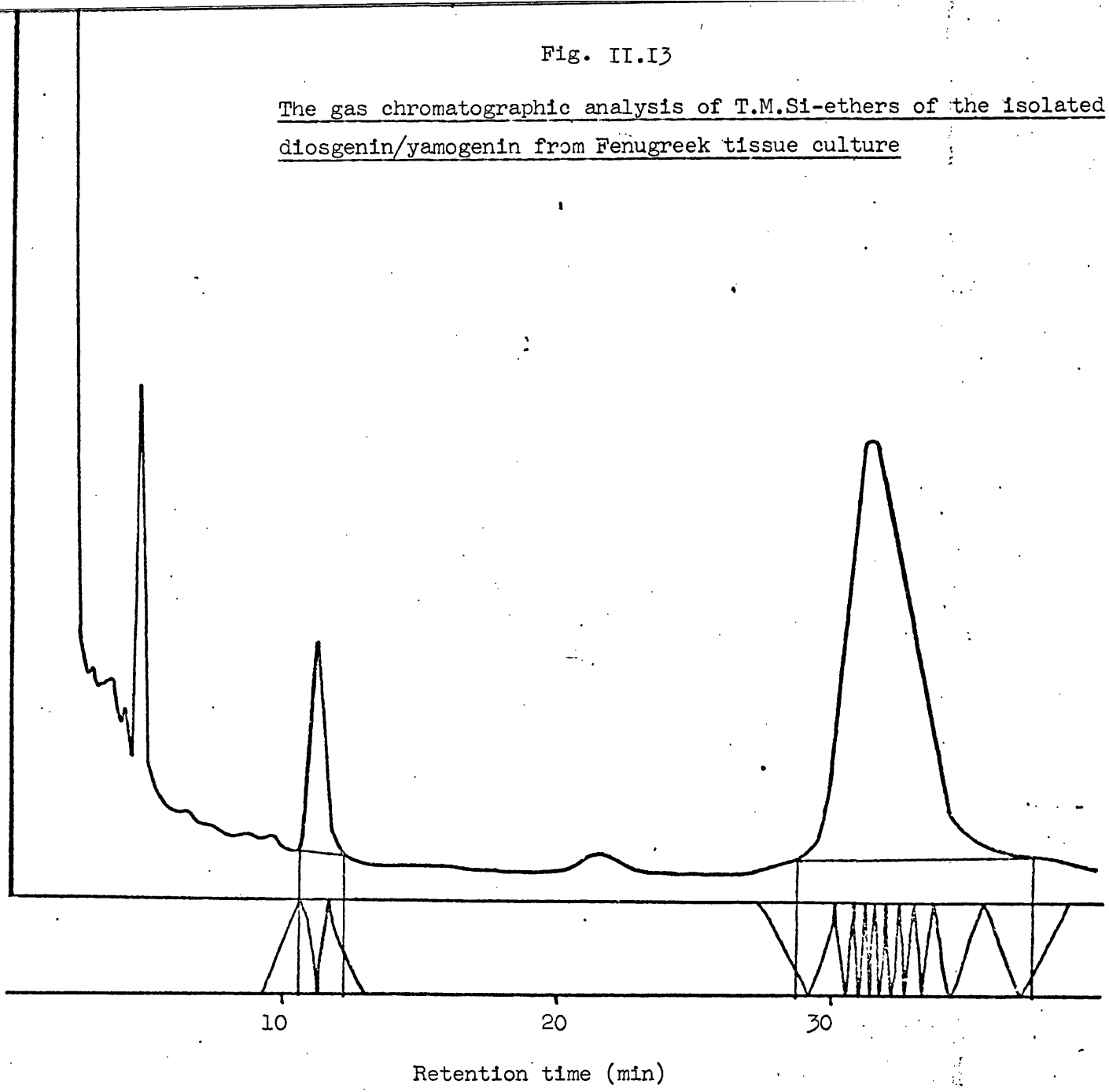
The relative retention time of the sapogenin mixture was similar to that of stigmasterol, Table II.21, and an infrared analysis was carried out to verify that the isolated fraction was sapogenin.

(4) The infrared analysis of the sapogenin fraction

Eddy[54] et al examined the complete spectra from 670-5000 cm^{-1} and concluded that the regions most useful for differentiating sapogenins were 700-1400 cm^{-1} and the carbonyl region from 1600-1800 cm^{-1}. Wall et al[55] obtained four absorption bands in the 1000-850 cm^{-1} region of the spectrum highly specific for the spiroketal side chain. All four bands at 865, 900, 920 and 981 cm^{-1} must be present for a steroidal sapogenin with an intact F ring to be present. The opening of the ring F results in the loss of the four bands associated with steroidal sapogenin. The 5-membered oxide ring E contributes little to the spectrum. The substitution of a hydroxyl into the C_{27} methyl group causes the four characteristic bands of the 25-R series, e.g. diosgenin to be absent. Substitution in the 25-S series, e.g. yamogenin,

Fig. II.13

The gas chromatographic analysis of T.M.Si-ethers of the isolated diosgenin/yamogenin from Fenugreek tissue culture



results in a strong band at 911 cm^{-1} and multiple bands at 960 cm^{-1} and 995 cm^{-1}.

Jefferies[52] analysed pure diosgenin and yamogenin and mixtures of the two. In the characteristic region 1050 cm^{-1} to 850 cm^{-1}, see Fig.II.I5, he found that pure yamogenin exhibited a stronger peak at 920 cm^{-1} than 900 cm^{-1} and in diosgenin the opposite applied. The 865 cm^{-1} band was sharp for pure diosgenin at 1% in chloroform, but fell as the proportion of yamogenin in the mixture increased. The 850 cm^{-1} band for yamogenin was small and irregular and disappeared at a D:Y ratio of 1:1. For quantitative estimations of diosgenin:yamogenin mixtures Wood[56] and Jefferies[52] both calibrated for yamogenin on the 920 cm^{-1} band and diosgenin on the 900 cm^{-1} absorption, the total steroidal sapogenin was calculated from the 980 cm^{-1} band.

In the experiments described in this thesis the T.L.C. and G.L.C. evidence of the presence of a mixture of diosgenin and yamogenin was confirmed by obtaining the spectrum of the fraction in the range 1050-850 cm^{-1}. A Hilger H800 double beam recording instrument with rock-salt prisms of 1 mm path length cell was used. A minimum concentration of 1% of sapogenin was required to obtain a response from the instrument and the volume of the cell was 0.5 ml. The minimum amount of sapogenin mixture required was therefore 500 µg.

Jefferies[52] had shown that the fixed oils, esters and sterol present in the crude extract from Fenugreek seed interfered with the infrared analysis of sapogenin mixtures and purified his extracts by adsorption column chromatography. Since the crude extract of tissue cultures was similar to

that from the seed, a column 30 cm x 1.5 cm diameter was packed with activity II silica gel, in a slurry with a solvent of hexane: ethyl acetate 9:1, to a length of 15 cm. A crude extract, obtained from 10 g of dried tissue, was transferred to the column in a total of 5 ml of hexane: ethyl acetate 9:1 and eluted with the same solvent. The sapogenin was collected in fractions from 150 to 210 ml of solvent (see Fig.II.I4). The use of only 9:1 reduced the amount of pigment eluted with the sapogenin. All traces of solvent were removed from the sapogenin fraction, which was dissolved in 0.5 ml of analar chloroform and transferred to the Hilger cell. The spectrum obtained is shown and compared with the spectrum obtained from the sapogenin of 2.5 g of seed RH.2336 obtained in the same way, see Figs.II.I5 & II.I6

(5) Diosgenin/yamogenin ratio of tissue culture and seed extracts

The ratio of diosgenin: yamogenin in the sapogenin mixture extracted from the callus tissue was compared with the sapogenin mixture from RH.2336 seed. In the callus the ratio was D:Y 52:48 and in the original seed 60:40.

(6) Mass spectrometric analysis of the sterol fraction

A bulked sterol fraction (approximately 30 mg) was purified by re-crystalisation from methanol and preparative T.L.C. A 20 mg fraction was recovered and the purity of the sample checked by converting 10 mg to the acetate derivative. The sample was run with a sitosteryl acetate standard, similarly prepared, on a 20% silver nitrate plate with benzene: hexane 1:1. No separation of the steryl acetate

Fig II.I T.L.C. of fractions obtained from a 15 X 1.5cm column activity II silica gel eluted with Hexane:Ethyl acetate 9:1.

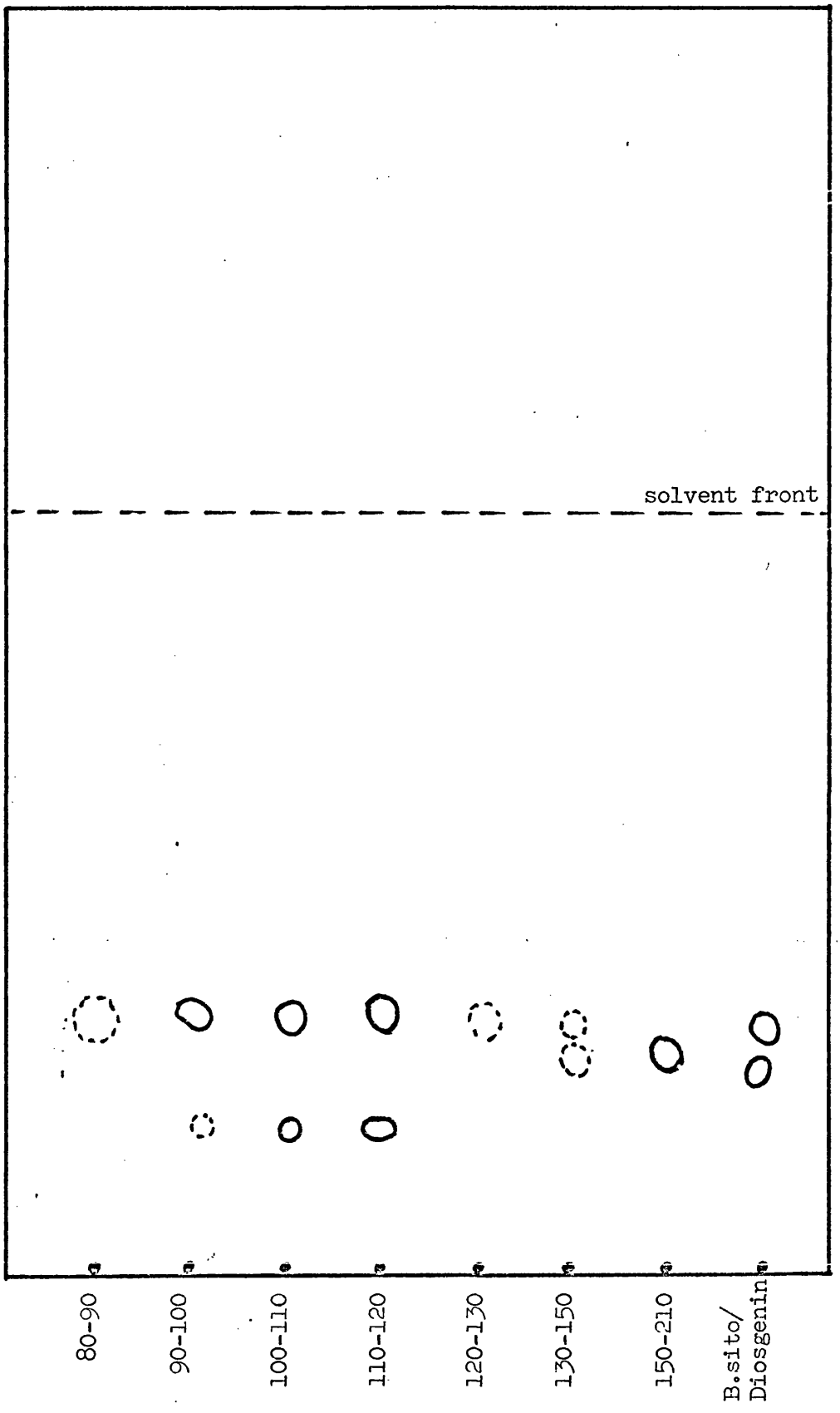
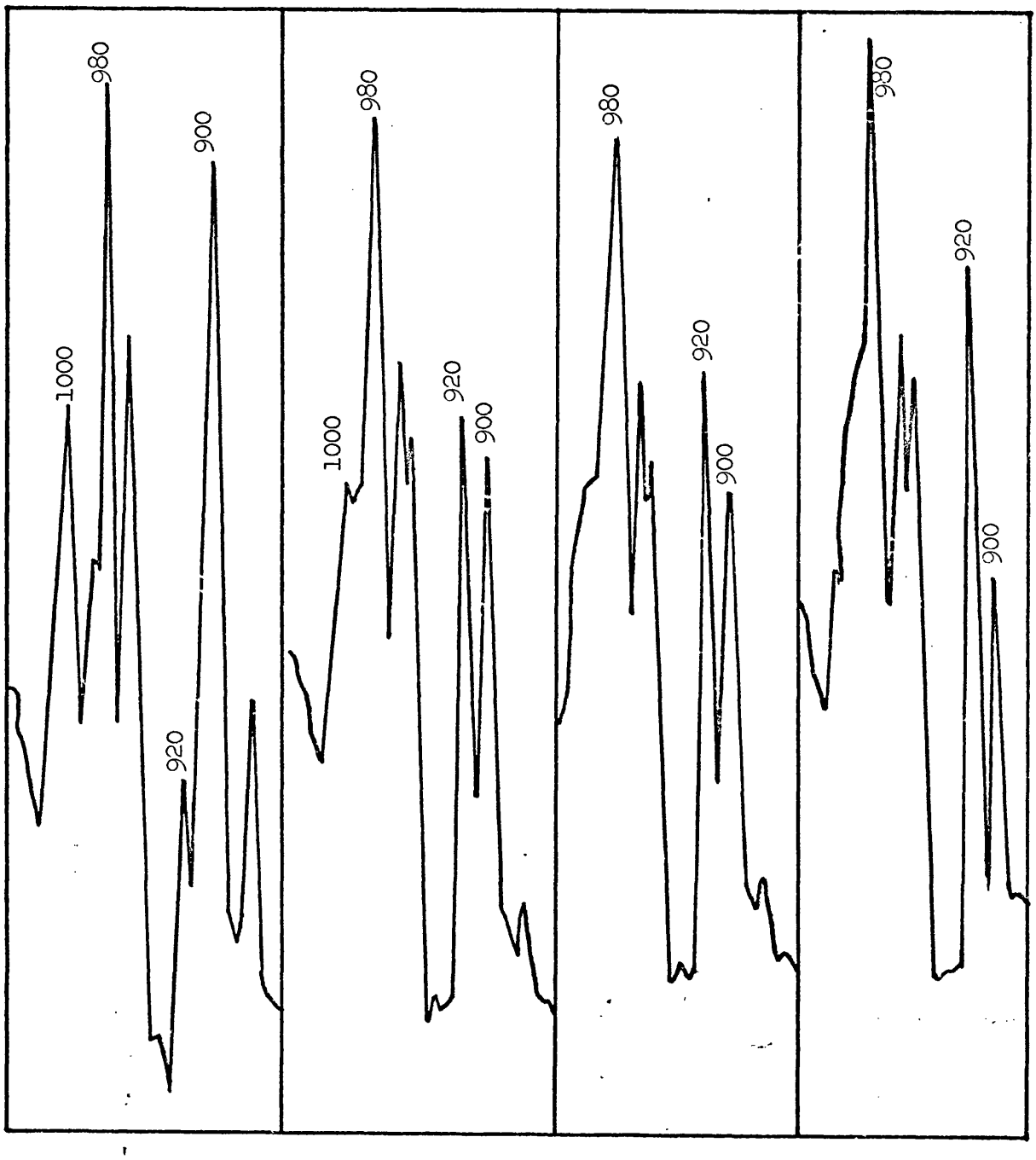
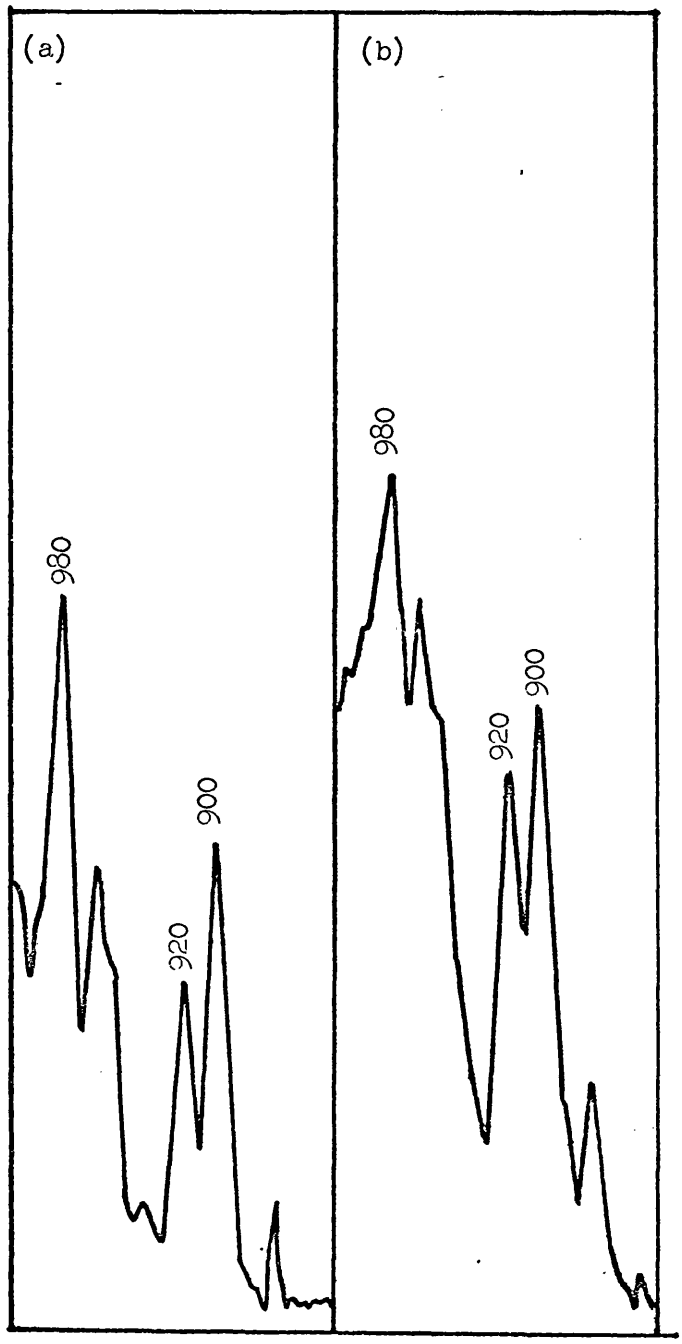


Fig II.15 The infrared spectra of chloroform solutions of :- a) diosgenin; b) diosgenin:yamogenin mixture 1:2 ; c) diosgenin:yamogenin mixture 1:3 ; d) yamogenin.



Reproduced from Ref :- 52

Fig II.16 The infrared spectra of chloroform solutions of diosgenin/yamogenin extracts of a) fenugreek seed; b) fenugreek tissue culture.



fraction occurred and the spot detected corresponded to the standard (Rf 0.42). G.L.C. analysis of the acetates showed the presence of all the peaks previously observed. The retention times relative to cholestane were cholesteryl acetate 2.38, campesteryl acetate 2.68, stigmasteryl acetate 3.02 and sitosteryl acetate 3.73. The T.M.Si-ethers of the sterol mixture was also obtained and showed the sample to be pure, see Fig.II.17 The sample was sent to the Tropical Products Institute for mass spectrometric analysis. The mass spectrum of the mixture contained molecular ions at 386, 400, 412 and 414 with their relative intensities being in the same order as the G.L.C. peak areas of A, B, C and D. Only the spectra of peaks C and D would be obtained individually using G.L.C./mass spectrometric techniques because of the low relative amounts of A and B present. Peaks C and D gave molecular ions at 412 and 414 respectively but comparison of the spectra with reference standards was not performed. The molecular weights of the two major peaks correspond with the molecular weights of stigmasterol and sitosterol and confirmed the earlier identification by G.L.C. retention times. From the presence of molecular ions at 386 and 400 in the mixture, coupled with the G.L.C. retention times of the minor peaks, it was thought that peak A was cholesterol and B was campesterol.

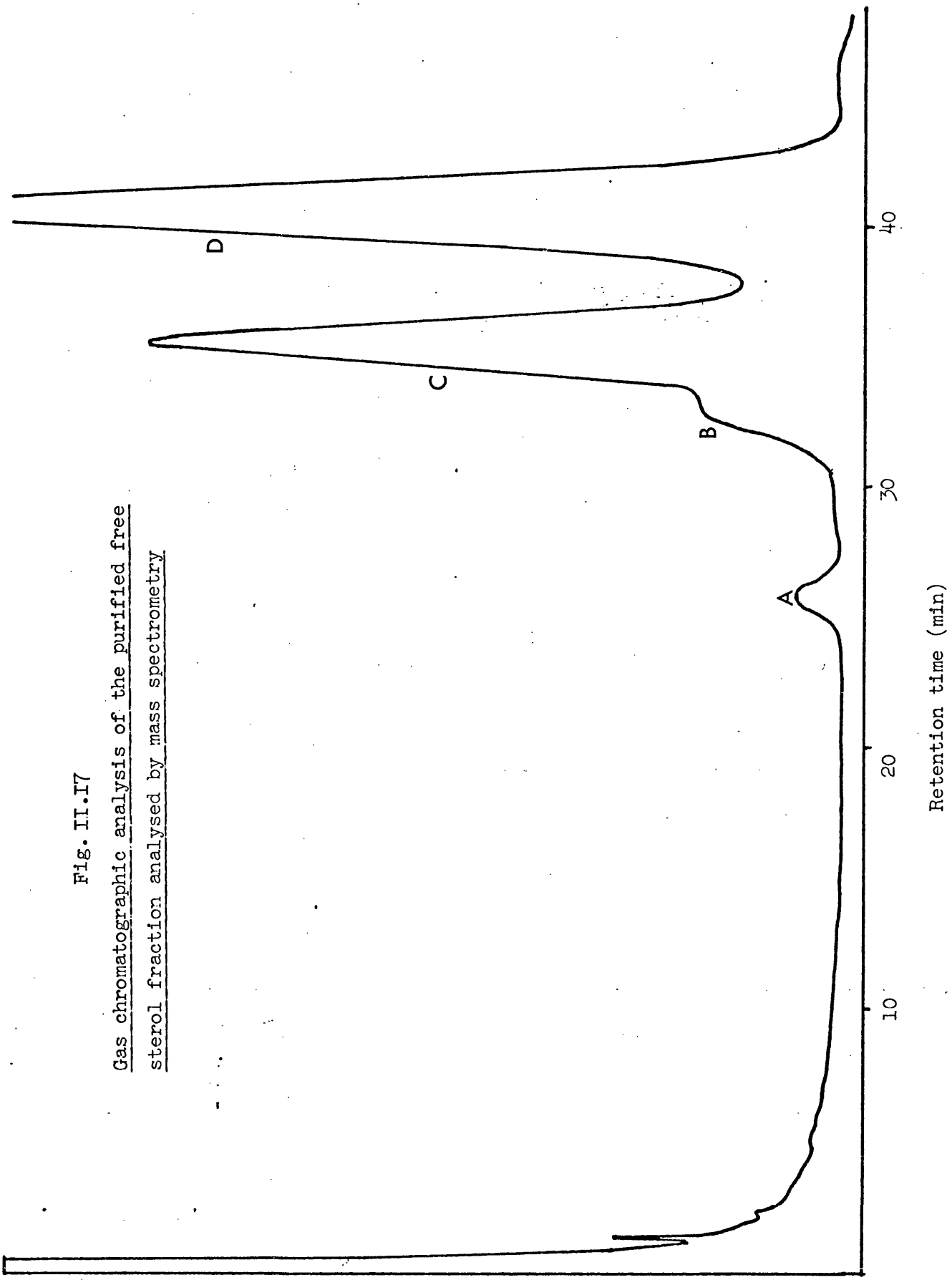


Fig. II.17

Gas chromatographic analysis of the purified free sterol fraction analysed by mass spectrometry

PART II CHAPTER IV

THE DETERMINATION OF SAPOGENIN

THE EXTRACTION OF STEROIDAL SAPOGENINS FROM PLANT MATERIAL

Much work has been done on the extraction of sapogenins from plant sources with special emphasis on the tubers of species of Dioscorea and the seeds of Fenugreek.

Earlier workers[57] extracted the saponins from powdered plant material with a polar solvent, such as ethanol. The ethanolic extract was defatted with ether and acid hydrolysed to release the water insoluble sapogenins. The sapogenin containing residue was made alkaline, dried and extracted with an organic solvent.

Rothrock, Hammes and McAleer[58] found the hydrolysis of the saponin within the powdered plant material was more practical. The subsequent extraction with organic solvent, after neutralisation and drying of the material, had fewer manipulative stages. In the extraction of diosgenin from the Dioscorea species the method provided a very pure diosgenin extract and they found that pulverised tuber hydrolysed with 2N acid for 2 hours gave the highest yield. Any increase in the hydrolysis time, or strength of the acid, reduced the amount of diosgenin extracted.

It has been shown that spirosta-3,5-diene compounds are formed by dehydrogenation of the corresponding spirostanols during acid hydrolysis of the saponins[59].

Yamauchi[60] determined the conditions under which spirosta-3,5-diene was formed from free diosgenin and a diosgenin glycoside, dioscin. He concluded that for the hydrolysis of the free glycoside, 4N aqueous sulphuric or 2N hydrochloric

acid were the mildest reagents, producing the least diene. The same worker found a greater tendency for the formation of spirosta-3,5-diene from the glycoside, than from free diosgenin. The formation of diene was accentuated by the use of ethanolic acid.

Blunden, Hardman and Morrison[61] confirmed the findings of Rothrock et al[58] concerning the optimum conditions of hydrolysis and extraction from fresh tubers. From the difference of the diosgenin content obtained by densitometric TLC, and the value for diene plus diosgenin obtained by infrared analysis, they calculated that 5 to 7% w/w of spirosta-3,5-diene was produced during hydrolysis of Dioscorea tuber.

The extraction of sapogenin from whole Fenugreek seeds has been carried out by several workers.[52 59 62 63] Bedou et al[59] hydrolysed the seed with 4N HCl for 3 hours and obtained substantial amounts of spirosta-3,5-diene. Fazli and Hardman[62] used 2N HCl for 2 hours on the whole seed and calculated that only 3% of the sapogenin was converted to diene. Miller[63] and Jefferies[52] both confirmed that the conditions employed by Fazli and Hardman[62] gave the optimum yield of diosgenin from intact Fenugreek seeds.

The need to neutralise acid insoluble residues after hydrolysis was shown by Rothrock et al[58] and Blunden and Rhodes[111] to be necessary to stop decomposition of the sapogenin during drying and storage. Sofowora[64] found 10% ammonia solution to be the most satisfactory neutralising reagent and Miller[63] and Jefferies[52] adopted the same reagent.

The extraction of sapogenins from tissue cultures has been reported by several workers. Kaul and Staba[50] refluxed the dried cells of Dioscorea deltoidea callus for four hours in

30% v/v HCl. The acid hydrolysed residue was filtered and washed with water until the filtrate pH was approximately 7. The washed residue was dried at 60°C for 8 hours and extracted with chloroform or benzene. Vagufalvi et al[65] extracted diosgenin from Solanum laciniatum tissue cultures. The method involved the acid hydrolysis of dried saponin obtained by extracting the powdered callus with methanol. Brain et al[38] hydrolysed the defatted, dried callus of Fenugreek with 2N HCl for 2 hours and extracted for 24 hours with light petroleum. Tomita et al[34] refluxed dry Dioscorea tokoro callus with 5% w/v HCl in 70% ethanol for 5 hours. Water was added to the ethanolic extract and sapogenin shaken out with ethyl acetate. Khanna[37] adopted a similar procedure for a quantitative determination of sapogenin in Fenugreek callus.

The method of Fazli[66], also adopted by Jefferies[52], using 2N hydrochloric acid for the hydrolysis and light petroleum for the extraction, was simple and produced small amounts of spirosta-3,5-diene. The hydrolysis conditions of Kaul and Staba[50] appeared too severe when considering the results of earlier workers.[60 62] Hydrolysis in the presence of ethanol was not considered suitable for the same reason.[60]

During the design of the assay procedure, and the initial attempts to screen developing tissue cultures for sapogenin, the hydrolysis and extraction procedure of Jefferies[52] was used. In the absence of sufficient cell cultures to carry out trials it was the most appropriate method. The method was later checked and the hydrolysis conditions, (2N hydrochloric acid refluxed for 2 hours) were not changed although the solvent of extraction of the steroids was changed from light petroleum to chloroform. It was found necessary to add about

1G of Kieselguhr to the neutralised, hydrolysed residue before oven drying to prevent caking and subsequent poor extraction.

THE QUANTITATIVE DETERMINATION OF STEROIDAL SAPOGENIN

(a) Infra-red spectrophotometry

Many methods have been devised for the assay of steroidal sapogenins in crude extracts from plants. The measurement of peak height in infra-red spectra has been used by several workers. Wall, Eddy, McLennan and Klump[55] used an IR procedure in the screening of plants for the presence of sapogenin. The extraction which involved the acid hydrolysis of alcoholic extracts defatted with benzene was long and contained inherent errors. Brain, Fazli, Hardman and Wood[67] used a method involving the acid hydrolysis of the plant material and IR assay of the crude extract. This gave a reliable assay when applied to Dioscorea tubers. Jefferies[52] found that removal of fixed oil and phytosterol from the crude extracts was necessary when assaying Fenugreek seed sapogenin and modified the method of Brain et al[67] to include a separation of the sapogenin from sterol and oil by adsorption chromatography. The method was highly reliable for extracts from 2.5g seed samples containing approximately 25 mg of sapogenin. The determinations were made on a Hilger H800 double beam recording instrument fitted with rock-salt cells of 0.4 ml volume. The minimum concentration of diosgenin/yamogenin required for a satisfactory spectrum was 2.5 mg/ml or 1 mg made up to cell volume. The normal working concentrations for the assay were 4 mg/ml for seed extracts. The amount of dried tissue culture material required to provide 4 mg of sapogenin would have been

approximately 20-25 g dry weight or 40-50 g for duplicates. This is equivalent to 500-600 g fresh weight and the facilities for growing this quantity for each of a series of cultures in an experiment were not available.

(b) Thin layer densitometry

Thin layer chromatography coupled with the scanning photo-electric densitometer was used in a technique developed by Blunden, Hardman and Morrison[68] for the assay of diosgenin in Dioscorea tubers. A linear relationship was shown to exist between the logarithm of the weight of sapogenin applied, and the square root of the absorbance of the spot produced. The experimental error of the method was found to be 7%.

The error of the assay was greatly reduced by the development of a template and syringe assembly which improved the plate spotting technique, (Brain and Hardman[69]), coupled with the repeated determination of sample spots. Jefferies found that the method was satisfactory for Dioscorea extracts but required modification of the TLC solvent system for use with the complex extracts from Fenugreek seed. The method is, however, slow and Jefferies[52] was only able to assay four samples a day and looked for a quicker alternative method.

Attempts were made to use the method for the determination of sapogenin from tissue culture extracts. It was found that the low sapogenin content in relationship to other components, especially phytosterol, made it impossible to obtain clearly defined and separated sapogenin spots at a concentration large enough for densitometric determination, without overloading the plate with other components. Later work showed the ratio of sapogenin:sterol to be 1:4 in hydrolysed culture extracts compared with 10:1 in seed and the method was not considered

applicable to the assay of tissue cultures.

(c) UV spectrophotometry

Ultra-violet spectrophotometry had been used by Walens, Turner and Wall[70] for the detection and estimation of steroidal sapogenins. The sapogenin was treated with 94% sulphuric acid at 40°C for 16 hours prior to estimation. Purification of the sapogenin was necessary and multi-component samples required fractionation into single, or two-component, mixtures before assay. The number of steps required to achieve pure sapogenin from crude Fenugreek extract would have made the assay highly inaccurate.

For extracts of tissue culture an assay procedure must be sensitive to small amounts of diosgenin, preferably without the removal of the sterol. Both colourimetric spectrophotometry and gas liquid chromatography are sensitive to small amounts of compounds. A colourimetric method was available in which the presence of phytosterol did not interfere with the reaction.

(d) Colourimetry

Yamagishi and Nakamura[71] assayed colourimetrically sapogenin extracts of species of Dioscorea after acid hydrolysis of the extracted saponins and preliminary separation of the components by a Florisil column. Diosgenin was estimated by a reagent prepared by dissolving 26 g of antimony trichloride in 5 ml of nitrobenzene and diluted with 1/10th its volume of 50% MeOH immediately prior to use. After heating at 60°C for 20 mins the solution was cooled and the optical density at the λ max of 500 nm was measured.

For the assay of tokorogenin [(25R)-5β-spirostan-1β2β 3α-triol] a reagent of antimony trichloride mixed with melted phenol in a ratio of 4:5 and with a λmax of 560nm was used.

The method was found to be sensitive enough to detect 1% diosgenin in a 10 mg sample (100 µg).

Sanchez et al[72], also working with Dioscorea, acid hydrolysed the saponin in the plant material and isolated the sapogenin by solvent extraction and thin layer chromatography. The diosgenin was extracted from the silica gel of the TLC plates with methanol. After filtration through a sintered glass funnel, the diosgenin was dried and treated with a reagent of sulphuric acid-methanol 80 + 20. The resulting chromophore was determined spectrophotometrically at λ_{max} 405 ± 1nm against a blank after 2 hours, which was the optimum time required for the chromophore to develop a stable optical density.

Slack and Mader[73] used 70-72% perchloric acid which gave a yellow chromophore with diosgenin and a λ_{max} of 408nm in the assay of Dioscorea extracts that consisted almost entirely of diosgenin. Miller[63] modified the procedure for the determination of diosgenin plus yamogenin in partially purified extracts from Fenugreek seeds.

THE COLOURIMETRIC ASSAY OF DIOSGENIN AND YAMOGENIN IN FENUGREEK TISSUE CULTURES

The extracts of Slack and Mader[73] consisted almost entirely of diosgenin with traces of yamogenin, gentrogenin [(25R)-spirost-5-en-3β-ol-12-one] and correllogenin [(25S)-spirost-5-en-3β-ol-12-one]. Miller[63] found that the plot of absorbance increase against concentration obeyed Beer Lambert's law for pure isomers of both diosgenin and yamogenin

and mixtures of the two. Miller was therefore able to determine the total sapogenin content of seed extracts which were known to contain the two isomers in the ratio of approximately 3:2, as determined by the absorption column and IR method of Hardman and Jefferies.[74] It was found that the crude extract of seed contains phytosterol to sapogenin in the ratio 1:10 whereas in the tissue culture extracts this ratio was 4:1. The perchloric acid formed no chromophore with phytosterol or gitogenin, but a colour reaction occurred with the spirostadiene formed during hydrolysis. Miller[63] removed this compound and the fixed oil, which also interfered with the colour reaction, by adsorption column chromatography.

(a) Adsorption column chromatography of crude extracts

Thin layer chromatography of crude sapogenin extracts from seed, on silica gel G plates with hexane:ethyl acetate 4:1, showed that fixed oil and spirostadiene ran near the solvent front and diosgenin had an Rf value of 0.27 Netting[75] showed that the properties of silica gel are the same in layers and columns and hexane:ethyl acetate 19:1 was found by Miller[63] to remove fixed oil and diene from a column and leave the other components of the fraction adsorbed on the silica gel. No further separation of components was required and a very polar solvent was used to remove the extract as one fraction from the column.

For 2.5 g seed samples Miller[63] used a column of internal diameter 1 cm packed with 6g of activity I silica gel as a slurry in hexane:ethyl acetate 19:1. The crude extract was transferred to the column quantitatively/with a total of 10 ml of the same solvent. A further 90 ml of 19:1 was used to remove fixed

oil and spirostadiene and this was followed by 10 ml hexane: ethyl acetate 1:1 and 50 ml of pure ethyl acetate to remove phytosterol plus sapogenin.

Insufficient quantities of tissue culture cells were available for use in the design of an assay and small quantities of seed extract were used to represent tissue extracts. A 2.5 g seed sample extract was diluted to 50 ml in petrol and 1 ml quantities containing approximately 500 µg of diosgenin/yamogenin used in experimental work. It was found that, with a column of i.d. 0.5 cm packed with silica gel Activity II to a height of 24 cm, all the fixed oil and diene and some phytosterol could be removed with 30 ml of hexane: ethyl acetate 9:1. A further 16 ml of hexane:ethyl acetate 1:1 completely removed the diosgenin/yamogenin.

(b) Colourimetric assay

Miller made the extract from a 2.5 g seed sample up to 5 ml with chloroform and added 5 ml of perchloric acid to a 20 µl aliquot, containing approximately 100 µg of diosgenin/yamogenin. The assay for tissue culture extracts was modified so that for a 2 g callus sample the sapogenin fraction made up to 5 ml with chloroform and aliquots of 1 ml were taken for assay. A 5 ml volume of perchloric acid was added to the sapogenin after removal of the chloroform by evaporation. The sapogenin and acid were agitated and centrifuged to remove a scum which tended to form, by depositing the scum on the upper part of the centrifuge tube. The determination was carried out after a total reaction time of 10 minutes and the absorbance was measured at 408 nm against a reagent blank. Determinations were made on a 3 ml aliquot of the perchloric acid

solution (from the centrifuge tube) in a 1cm silica cuvette using an SP600 spectrophotometer.

(c) The calibration curve for the determination of Diosgenin and Yamogenin by the colourimetric method

Miller[63] obtained linear relationships for the increased absorbance with concentration for both isomers. He was able to determine the ratio of the two isomers in seed extracts from their infra red spectra and obtained a linear response for the isomers in the ratio found in seed extracts. The ratio of diosgenin/yamogenin present in tissue cultures was not known when the procedure was used, because no material was available for IR investigation. A calibration curve was therefore prepared using a diosgenin yamogenin mixture in the ratio found in the seed from which the tissue cultures were initiated (D:Y 3:2).

A series of standard solutions were prepared and four determinations were made per standard. The mean absorbance value for each standard was calculated and a calibration curve prepared. The results are shown in Table II.22 and the calibration curve in Fig. II.18

Table II.22

Preparation of a calibration curve for the determination of Diosgenin/Yamogenin by the Colourimetric Assay

Diosgenin + Yamogenin mg	Mean Absorbance
10	.0491
20	.0899
30	.1349
40	.1884
60	.2807
80	.4078
100	.4711

Slope = .0048829

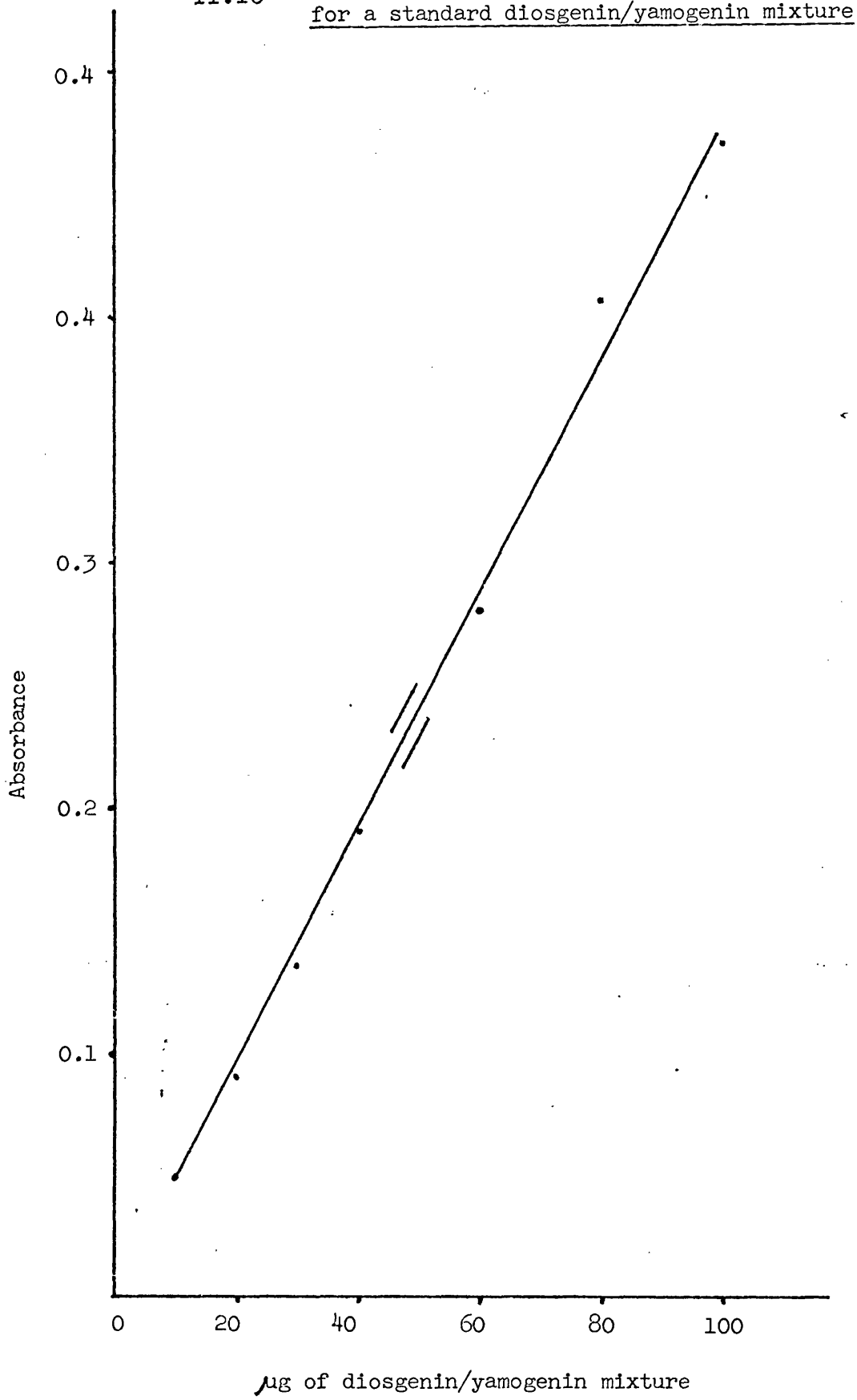
Confidence Interval

Syx^2 = .0001528

Sy^2 = .0000218

Sy = .00467

Fig. II.I8 The colourimetric assay calibration curve for a standard diosgenin/yamogenin mixture



(d) Estimation of the error of the column recovery and colourimetric determination procedure

A standard solution containing 100 µg of diosgenin/yamogenin standard and 1 mg of acid hydrolysed, sapogenin free, Fenugreek oil, per ml was prepared in chloroform. A 5 ml aliquot of this solution was added to each of ten 100 ml round-bottomed flasks and evaporated to dryness to simulate a petrol extract. The sapogenin was separated from the fixed oil by column chromatography and colourimetrically assayed. It was found that the volumetric flask used to adjust the column separated sapogenin extracts to volume contained 4.94 ml and not 5 ml. The final theoretical total sapogenin recovery was accordingly 101.2 µg/ml. Two colourimetric assay determinations were carried out for each of the ten samples. From the mean absorbance value the sapogenin present was calculated.

Mean Absorbance	Diosgenin/Yamogenin µg	% Recovery
.4875	101.0	99.8
.4730	98.0	96.8
.4875	101.0	99.8
.4750	98.3	97.1
.4980	103.0	101.8
.4830	100.0	98.8
.4900	101.5	100.3
.4925	102.5	101.3
.4680	97.0	95.8
.4925	102.0	100.8

The mean recovery value for the sapogenin was 99.24%. Estimation of the error for the method gave a value for the sapogenin, at $p = 0.05$, of 100.4 µg $\pm$ 4.1% and for duplicate results, as employed in the assay, 100.4 µg $\pm$ 2.89%. This

value was the error for only the column separation and spectrophotometric determination and not for the complete extraction and assay method.

MODIFICATION OF THE PERCHLORIC ACID COLOURIMETRIC ASSAY

The foregoing assay did not prove satisfactory when applied to established tissue cultures of Fenugreek. The results of the assays of freshly initiated tissue cultures show a rapid fall in their diosgenin/yamogenin content compared with the content of the original cotyledons. Thin layer chromatographic examination did not indicate a similar fall in the other components of the crude extracts. When the sapogenin level fell below 0.07% m.f.b. it became impossible to obtain reliable duplicates. In these circumstances other components of the partially purified extract were thought to be interfering with the colour reaction. Reliable duplicates could be obtained for low concentration of pure standard. The chlorophyll produced by the tissue grown in continuous light also interfered with the spectrophotometric determination. Green pigment was eluted with the sapogenin fraction during column separation and addition of perchloric acid to such extracts resulted in the formation of a thick brown scum. Even after centrifugation, it was difficult to avoid transferring some of the scum to the cuvette with the perchloric acid.

(a) Modification of the column separation procedure.

The length of the column was altered from 24 to 31 cm of Activity II silica gel and fixed oil and most of the phytosterol were eluted with 35 ml of hexane:ethyl acetate 19:1.

Sapogenin was recovered with a further 30 ml of the same solvent. This system reduced the amount of chlorophyll eluted with the sapogenin.

(b) Estimation of error for the modified colourimetric method

The error for the complete assay, including modified column separation, was calculated. Sufficient dried tissue culture was available to allow the overall error of the assay, including acid hydrolysis and petrol extraction to be found.

The dried tissue was powdered, sieved and mixed thoroughly to provide a uniform sample. Nine aliquots were taken and extracted by the method used previously. After adsorption column chromatography, the diosgenin/yamogenin content was determined, Table II.23

The mean value for the nine samples assayed was 354 ug/g $\pm$26.62%. For duplicate determinations the value was 354 $\pm$18.82% at p = 0.05.

The experimental error for this method was unacceptable and the method was not used for further assays.

Table II.23

Estimation of error for the modified Colourimetric Method

Sample	Sample wt.	% m.f.b. Diosgenin + Yamogenin
1	1.0646	.0394
2	0.9790	.0385
3	1.1776	.0341
4	1.1952	.0265
5	1.0884	.0382
6	1.1364	.0340
7	1.0388	.0420
8	1.0916	.0312
9	0.5946	.0349

Mean % m.f.b. = 0.0354%

Coefficient of variation = 13.31

The percentage range of error at p = .05 = $\pm$ 26.616%

" " for duplicates = $\pm$ 18.82%

THE DETERMINATION OF DIOSGENIN/YAMOGENIN IN COTYLEDONS DURING THE INDUCTION OF TISSUE CULTURES

An experiment was carried out to monitor the diosgenin content of Fenugreek cotyledons during the induction of tissue cultures. A sample of Ethiopian seed RH.2602 was surface sterilised and placed on 1% agar in distilled water and germinated in the dark. After 12 hours the viable seed had absorbed water and swollen. The cotyledons were aseptically removed from the swollen seed and cultured on MS medium containing 10 ppm NAA and 10% v/v coconut water. After 3 days growth in continuous light the cotyledons had turned green and after one week undifferentiated cell growth had occurred at the cut edges of the cotyledon.

Assays were carried out on samples harvested on the first, third, seventh and twentieth day of growth. Thin layer chromatography of the sapogenins showed, as in the case of the whole seed, that the predominant sapogenins were diosgenin and yamogenin.

The assay results, TableII.24, show a high initial concentration of diosgenin plus yamogenin of 3.38%, but after seven days when callus tissue had been formed by all the cotyledons assayed, the % w/w m.f.b. of sapogenin had fallen to nearly half the original value (1.98%), Fig.II.19 A further fall in sapogenin content in the cotyledons producing callus tissue was recorded after twenty days. The fall in concentration could have been due to

(i) a 'dilution' of the sapogenin present in the original cells by the active division and increase in the cell mass of the new tissue

Table II.24

The quantitative determination of Diosgenin/Yamogenin in cotyledons during the induction of tissue cultures

Days of Growth	% m.f.b.	Mean m.f.b.	Diosgenin/ Yamogenin % m.f.b.	Mean Diosgenin/ Yamogenin % m.f.b.
1	39.5 41.8	40.65	3.74 3.94	3.84
3	29.1 27.8	28.45	3.32 3.43	3.38
7	18.6 18.8	18.71	1.90 2.05	1.98
20	9.4 11.8	10.60	0.93 0.84	0.88

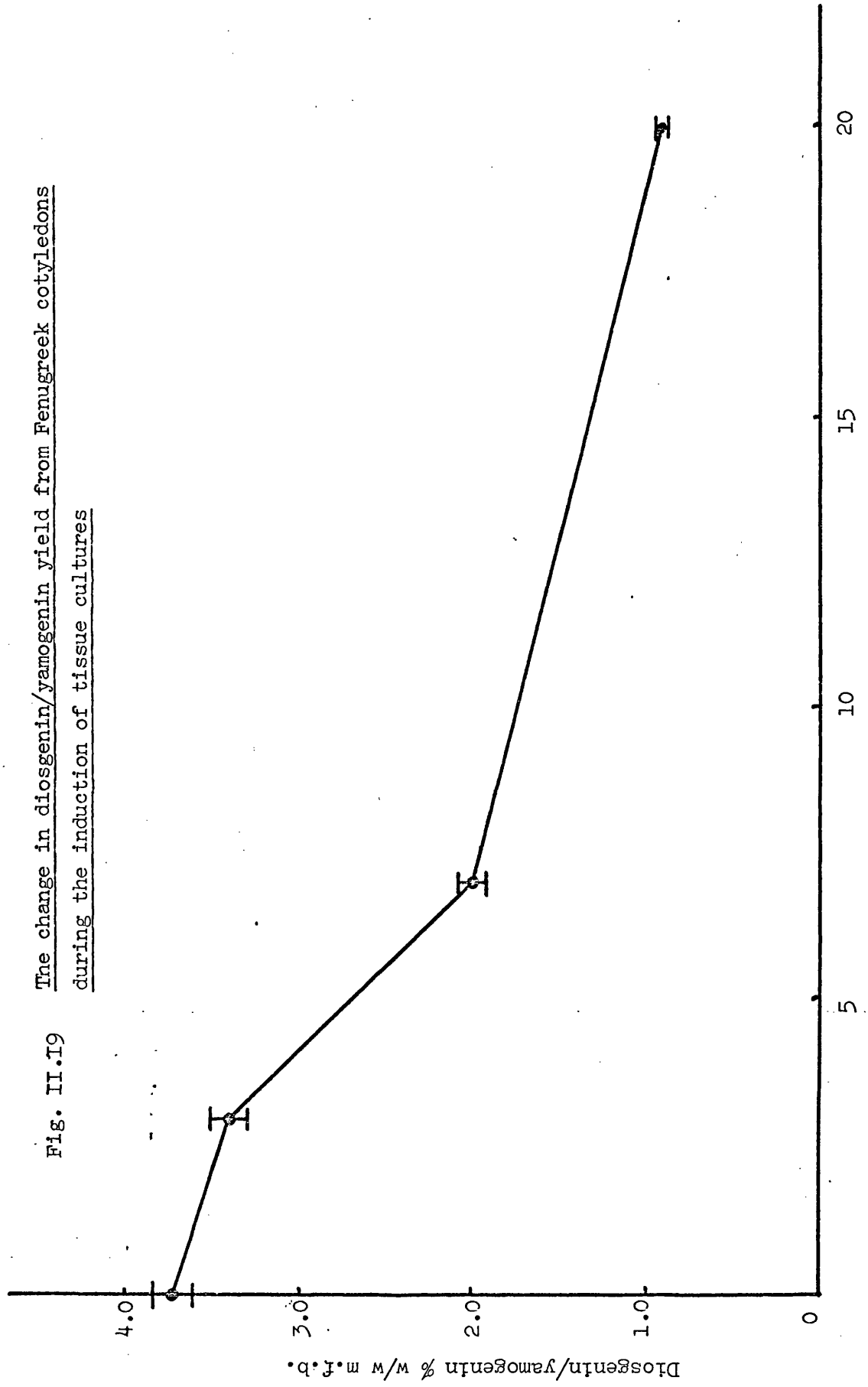


Fig. II.19 The change in diosgenin/yamogenin yield from Fenugreek cotyledons during the induction of tissue cultures

(ii) utilization of saponin in furostan form as available sugar and sterol, the latter by opening of ring E to afford 16,22,26 trihydroxy cholesterol available for metabolism by the plant cells. This would be a reversal of the biosynthetic route of sapogenins as demonstrated in tissue cultures of Dioscorea tokoro[76]

(iii) the diffusion of the saponin into the medium.

A second experiment was carried out to study the sapogenin level of tissue cultures over a longer period of time.

THE DETERMINATION OF DIOSGENIN/YAMOGENIN IN YOUNG TISSUE CULTURES INDUCED FROM FENUGREEK COTYLEDONS

The experimental procedure was modified to reduce the likelihood of contamination and the premature curtailment of the experiment which had occurred when using Ethiopian seed RH.2602. The seed RH.2336 Moroccan strain was chosen because it had a tougher seed coat than RH.2602 and surface sterilisation did not kill, or damage, the seed. The cotyledons were not removed from the soaked seed because the technique was impractical when dealing with large numbers of seed and was susceptible to contamination. Seed was allowed to germinate and grow for a period of five days on 1% agar during which time the cotyledons emerged from the seed, making aseptic removal easier. Sufficient seed was grown to allow immediate assay of a sample of cotyledons consisting of one of each pair removed from each seed. A second assay was carried out after 6 days when the first callus started to form, and a third assay

at one month after the first subculture. Subsequent assays were carried out on cells harvested at each subculture, Table II.25. The tissues were initiated from seeds germinated in total darkness and from these two sets of cultures were grown, one in permanent light and the other in permanent darkness. A marked similarity in sapogenin level and moisture content occurred during the growth of the two cultures over the first 106 days. As in the original experiment, the diosgenin/yamogenin level of the tissue fell rapidly in the first month. A similar rate of fall occurred in the second month of growth, in which the tissue cultures became established and grew rapidly. During the third and fourth months the level detected appeared to remain constant. This indicated that an equilibrium had been achieved between the rapid growth rate of the tissues and the biosynthesis of diosgenin/yamogenin. No significant difference was found in the diosgenin/yamogenin content of the two cultures after 106 days, Fig. II.20

In samples from subsequent months growth the assay results became unreliable. It became impossible to obtain consistent duplicate results. The perchloric acid reacted with the partly purified sapogenin fraction to produce a brown scum which interfered with the colour reaction and could not be displaced by centrifugation.

Attempts to assay the harvested cells by the perchloric acid method already described were stopped, but the tissues were maintained under the same conditions and continued to be subcultured at monthly intervals.

Table II.25

The determination of diosgenin/yamogenin in young tissue cultures induced from Fenugreek cotyledons

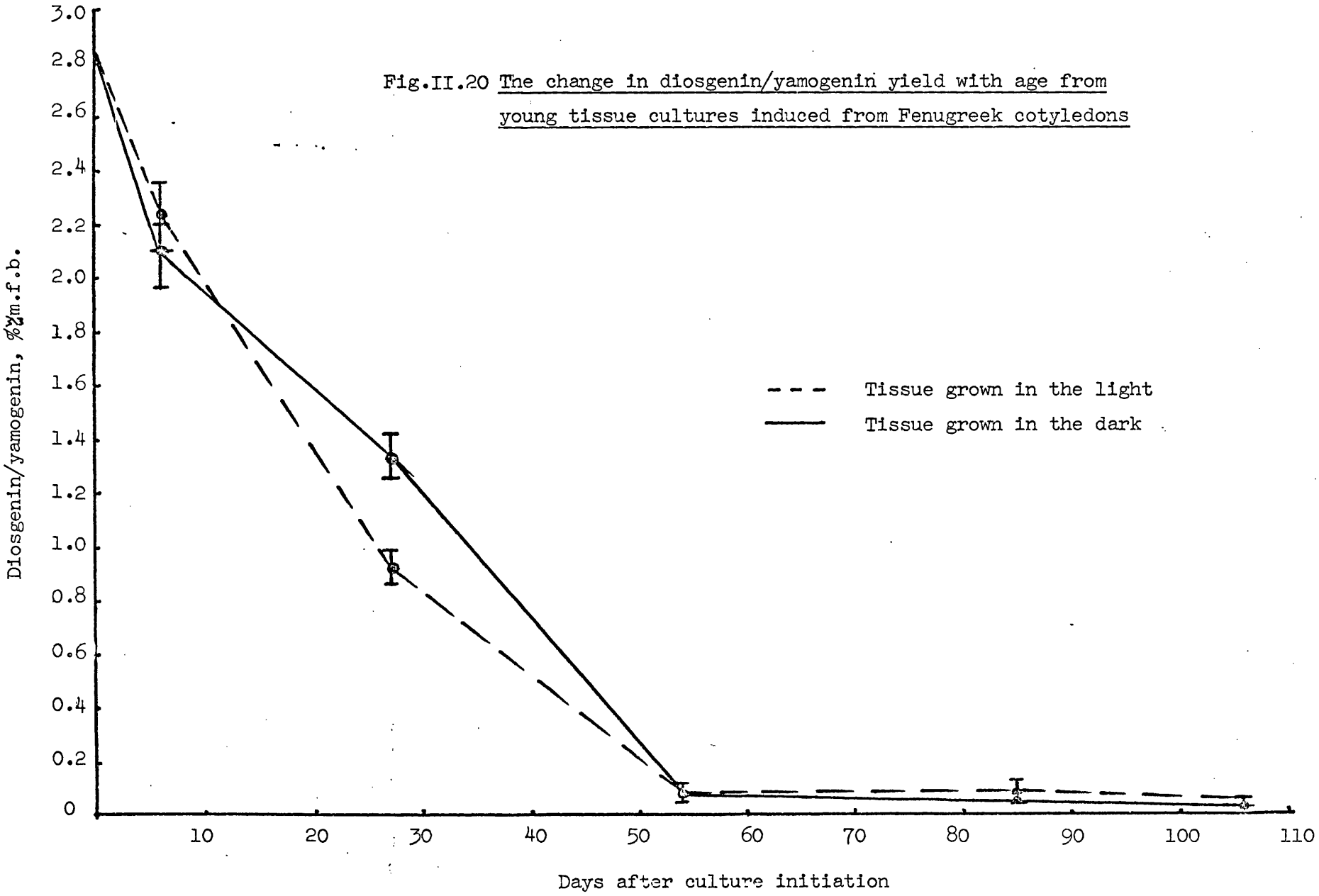
LIGHT

Days of growth	% m.f.b.	Diosgenin/ Yamogenin % m.f.b.	Mean m.f.b.	Mean Diosgenin/ Yamogenin % m.f.b.
1	18.6 19.1	3.20 2.45	18.85	2.825
6	16.8 16.1	2.11 2.34	16.45	2.225
27	12.2 13.2	0.87 0.97	12.70	0.920
54	7.9 8.7	0.08 0.09	8.30	0.085
85	9.1 7.9	0.08 0.09	8.50	0.085
106	8.7 8.9	0.05 0.06	8.80	0.055

DARK

Days of Growth	% m.f.b.	Diosgenin/ Yamogenin % m.f.b.	Mean m.f.b.	Mean Diosgenin/ Yamogenin % m.f.b.
1	18.6 19.1	3.20 2.45	18.85	2.825
6	13.7 13.7	2.09 2.09	13.70	2.090
27	12.6 11.5	1.50 1.17	12.05	1.335
54	7.3 7.2	.07 .07	7.25	.070
85	6.9 6.7	.04 .05	6.85	.045
106	6.8 7.6	.04 .02	7.20	.030

Fig.II.20 The change in diosgenin/yamogenin yield with age from young tissue cultures induced from Fenugreek cotyledons



After one years growth a faint spot corresponding to diosgenin standard and indicating a very low level of sapogenin was still detectable on thin layer chromatograms of tissue culture extracts from cultures grown both in continuous light and in continuous dark conditions.

PART II CHAPTER V

THE G.L.C. ASSAY OF SAPOGENIN AND PHYTOSTEROL

THE ASSAY OF DIOSGENIN/YAMOGENIN

The preliminary analysis of tissue cultures for phytosterol and sapogenin indicated the possible use of G.L.C. as a method of assay for both components. Cook[77] used G.L.C. for the determination of diosgenin in Dioscorea tubers. The method involved the same type of column as that used in our analyses (SE30). Another procedure was also devised by Rozanski[78] who again based his method on an SE30 liquid phase column.

Both methods involved the assay of crude extracts of material with a low level of minor components, which were unlikely to interfere with the determination. The method of Rozanski simply involved the partition of released sapogenin into an immiscible xylene layer during aqueous acid-hydrolysis. With no further purification the xylene extract was assayed.

With a 2½% SE30 column operating at 270°C and a helium carrier gas flowing at 60 ml/min Rozanski obtained no separation of diosgenin and yamogenin. The yamogenin formed a slight shoulder on the diosgenin peak. Other workers[52 77] were also unable to separate the two isomers and assayed the diosgenin plus yamogenin. Gas liquid chromatography has been used for the estimation of diosgenin in tissue cultures.[50] Crude chloroform extracts from Dioscorea tissue cultures were sufficiently pure for direct assay on a QF-1 (3%) column.

The feasibility of assaying crude extracts of Fenugreek seed by G.L.C. had previously been tested.[52] Samples were injected onto a 1 metre column of 5% OV101 on chromasorb G with cholestane as an internal standard. Relative retention times obtained were cholestane 1 (9.3 mins), diosgenin 2.84, yamogenin 2.92 and sitosterol 3.17. Analysis of a mixture of standard steroids in the proportions present in the seed was carried out. Sapogenin, as diosgenin and yamogenin 3:2, was mixed with sitosterol in the ratio 10:1. Jefferies[52] found that assay of the sapogenin was not accurate because of interference caused by the presence of the phytosterol.

Sitosterol was the major steroidal component of the extract of Fenugreek callus, being approximately four times the weight of the sapogenin. In callus extracts the presence of stigmasterol as a second major sterol peak also occurred. The retention of stigmasterol T.M.Si-ether, relative to cholestane was 2.76 and that of a diosgenin/yamogenin mixture 2.82. The use of a crude extract for the G.L.C. assay of sapogenin was, therefore, not possible and a method of purification of the sapogenin was designed for extracts from 2-3 g of callus.

(1) Adsorption column chromatographic separation of the sapogenin

In the colourimetric assay method, adsorption column chromatography had been used to obtain a partial separation of the phytosterol and sapogenin fraction of callus extracts.

A column of 31 cm length with a bore of 5 mm, packed with silica gel of activity II, provided a separation of phytosterol

and sapogenin, but the procedure was not reliable, being affected by small variations in flow rate. Although by doubling the solvent and time required to run a column, the use of activity I silica gel did provide a consistent separation. The amount of pigment and other compounds removed from the column was also reduced with activity I silica gel.

The final method adopted is shown in Table II.26

(2) G.L.C. conditions of diosgenin/yamogenin assay

The sapogenin fractions (isolated by the column chromatography method) were analysed as T.M.Si-ethers on the 6 ft SE30 column used previously. The analysis showed the presence of one peak corresponding to a diosgenin/yamogenin mixture and no trace of sitosterol, the major phytosterol component. A 2-3 g sample of dry tissue was found to provide sufficient sapogenin (about 200-500 ug) for a satisfactory analysis.

The retention time of the sapogenin was long with the 6 ft column, but it was used because the phytosterol fraction, which was also to be analysed, could not be separated on a 1 metre column.[78] The higher temperatures available to Rozanski through the use of helium carrier gas allowed a retention time for diosgenin of 7 minutes on a 2 metre SE30 column. After six months use, the maximum working temperature of the SE30 columns was increased from 250 to 255 with no apparent loss of stationary phase. This considerably reduced the retention time of sapogenin T.M.Si-ether to 31 minutes, compared with 27 minutes for a 1 metre OV101 column at 250°C used by Jefferies.[52] The carrier gas flow rate was not altered for sapogenin assay and remained at 60 ml/min.

Table II.26

Solvent sequence for the isolation of phytosterol and sapogenin from tissue culture extracts by adsorption column chromatography

Eluate composition	Solvent sequence	Eluate assessment
	5 mm x 31 cm Activity I 9:1 Hex:EtAc	
Spirostadiene		
Steryl esters		
Fixed oil	20 ml	
Sterol	55 ml	Collect
Sterol or D + Y	1x5 ml	T.L.C. check
	Hex EtAc 5:2	
Diosgenin + Yamogenin	10 ml	Collect
	1x5 ml	T.L.C. check

(3) The G.L.C. determination of diosgenin/yamogenin as T.M.Si-ether

A linear relationship had previously been obtained for T.M.Si-ethers of sitosterol by measuring peak area with a disc integrator.[52] In this work the relationship of free sitosterol over a range of concentrations gave a curve.

The disc integrator method was used to obtain a linear relationship for a diosgenin/yamogenin 3:2 standard mixture as T.M.Si-ether over the range 100 to 1100 ug of sapogenin, Table II.27 and the graph obtained is shown in Fig.II.2I

(4) Estimation of the sapogenin recovery from the adsorption chromatography column

The recovery of sapogenin from the adsorption column chromatography system used, was tested. A series of eight columns was packed and each column loaded with a mixture of a standard sample of diosgenin/yamogenin mixture and acid treated, sapogenin-free oil. Two ml of the mixture containing 2 mg of oil and 200 µg of sapogenin was added to each column.

The columns were eluted as previously described and the sapogenin collected assayed by G.L.C. as T.M.Si-ether, Table II.28 The same standard sapogenin mixture was used for both the recovery experiment and the calibration curve.
The recovery obtained was 99 to 104% giving a mean result of 201 µg, compared with the theoretical result of 200 µg, and a coefficient of variation of 2.1%.

Table II.27

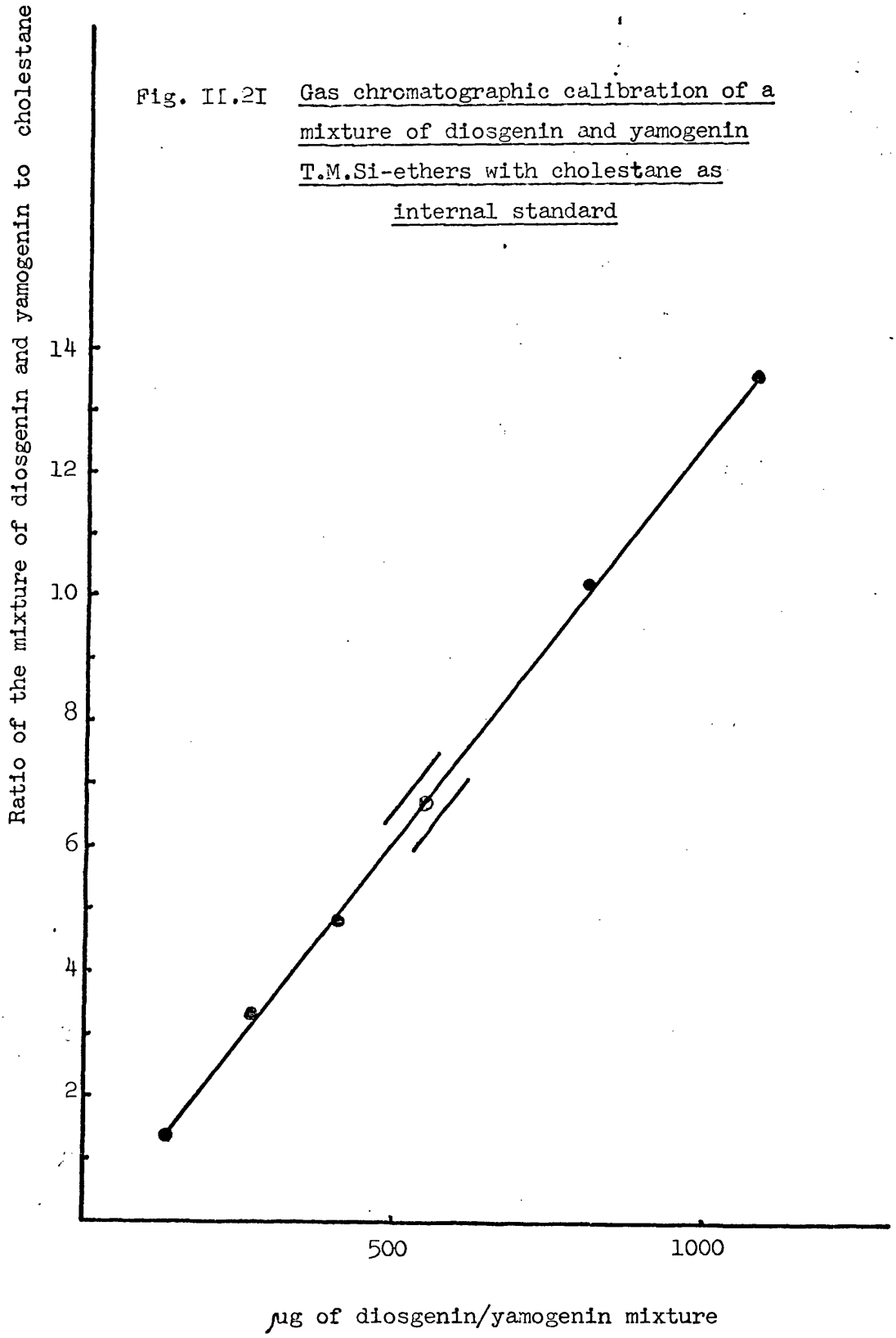
Gas chromatographic calibration of diosgenin/yamogenin as T.M.Si-ether

Injection size	Diosgenin in µg	Ratio diosgenin/yamogenin to cholestane		Mean
		1	2	
2.5 ul	135	1.45	1.51	1.48
2.5	270	3.20	3.41	3.30
2.0	405	4.65	4.97	4.81
2.0	540	6.42	6.84	6.63
1.5	810	10.04	10.41	10.22
1.0	1080	13.31	14.77	13.54

Slope = .0127

Confidence interval = 6.66 ± 0.49 or $6.66 \pm 7.3\%$

Fig. II.2I Gas chromatographic calibration of a mixture of diosgenin and yamogenin T.M.Si-ethers with cholestane as internal standard



TableII.28

The recovery of diosgenin/yamogenin from adsorption chromatography columns

Column	Sapogenin µg mean of 2 det.	% recovery
1	198.0	99.0
2	201.4	100.7
3	202.0	101.0
4	194.0	97.0
5	199.4	99.7
6	200.0	100.0
7	208.0	104.0
8	204.0	102.0

Mean Recovery 201 ug or 100.5%

(5) The development of the hydrolysis and extraction procedure for tissue cultures

In the usual procedure for seed, the sample was hydrolysed by refluxing with 2N hydrochloric acid for two hours. After filtration the acid insoluble residue was washed with 10% ammonia solution. The alkaline residue was dried at 60°C for 12 hours and extracted to exhaustion in a Soxhlet apparatus, with 40-60° B.P. petroleum spirit, for 24 hours.

Unlike whole seed, the hydrolysed residue from powdered callus formed a very hard lump when dried. It was found that this had to be powdered before extraction could take place. Powdering resulted in significant loss of material. It was found that the addition of approximately 1 g of hyflo supercel per 2 g of hydrolysed callus before filtration kept the dried residue porous.

Furthermore, re-extraction of callus-kieselguhr residues with chloroform, after the 24 hour petrol extraction, resulted in detectable amounts of diosgenin being extracted. Extraction with chloroform resulted in complete removal of diosgenin/yamogenin in 24 hours.

The nature of the material to be extracted by the method outlined was different from seed and tuber tissue. Callus tissue consists of undifferentiated, thin walled cells in a friable mass. The ease of acid penetration and also the chemical nature of the saponins to be hydrolysed might be different.

Experiments were carried out with a series of hydrolysis times with 2N hydrochloric acid on samples of dried tissue. The drying of fresh tissue produced irregular lumps of

material and these were powdered and mixed to provide a uniform sample for the hydrolysis experiments. After hydrolysis and extraction the diosgenin was assayed by the G.L.C. method already described.

The results for the first experiment (Table II.29 and Fig.II.22) show an incomplete hydrolysis after 30 minutes with a maximum yield occurring at 1½ hours. A second experiment was carried out with hydrolysis times extended to six hours. In this experiment the yield fell after four hours. The results show the mean of two determinations at each time. A time of two hours was adopted for the hydrolysis of tissue culture material.

(6) The determination of error for the extraction and G.L.C. assay procedure

The overall error of the assay procedure including the acid hydrolysis, chloroform extraction, adsorption column separation and G.L.C. was tested. A series of tissue cultures were dried, powdered, sieved and thoroughly mixed and ten aliquots of 2.5 g were taken and assayed for diosgenin plus yamogenin. The mean value for diosgenin/yamogenin was 150.3 µg/g ± 10.75%, Table II.30 For duplicate results, as employed in the routine analysis of tissue samples, the corresponding error was calculated to be 150.3 ± 7.6%.

Table II.29

Diosgenin/yamogenin recovery from callus hydrolysed with 2N hydrochloric acid for different times

	Mean of two determinations	
Time in Hours	% m.f.b. Diosgenin/ Yamogenin	% m.f.b. Diosgenin/ Yamogenin
½	.0172	
1	.0221	.0096
1½	.0223	.0098
2	.0215	.0109
3		.0107
4	.0200	.0082
6		.0069

Fig. II.22 The diosgenin/yamogenin recovery from tissue cultures (two samples) hydrolysed with 2N HCl for different lengths of time

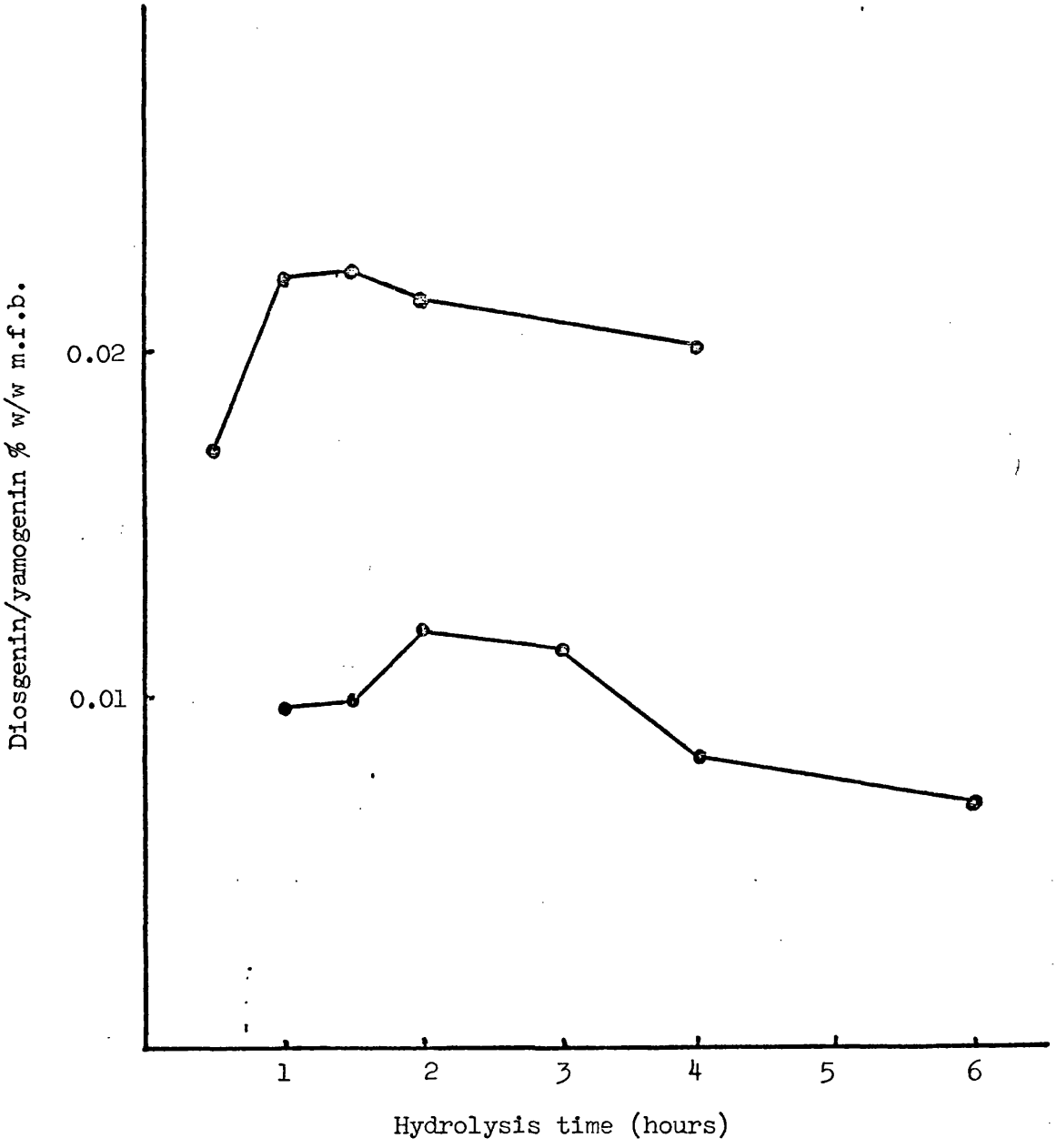


Table II.30

The determination of error of the diosgenin/yamogenin assay procedure

Sample wt. - g.	Mean ratio relative to cholestane	Wt of diosgenin/yamogenin in µg per g of callus	% m.f.b.
2.8955	5.75	163.0	0.0163
2.3299	4.00	146.0	0.0146
2.3294	4.52	163.0	0.0163
2.4020	4.31	151.0	0.0151
2.5578	4.65	152.5	0.0152
2.5621	4.50	147.5	0.0147
2.7103	4.71	145.5	0.0145
2.8280	5.05	148.5	0.0148
2.6245	4.52	145.0	0.0145
2.4704	4.12	142.0	0.0142

Coefficient of variation = 4.84%

The percentage range of error for duplicate determinations at P = 0.05

$= 150.4 \pm 7.6\%$

THE ASSAY OF THE FREE PHYTOSTEROL

The G.L.C. method used for the assay of sapogenin was adapted for the assay of the free phytosterol. The assay involved the direct determination of one of the major phytosterol components, sitosterol. Other components in the fraction were estimated by comparison of their peak areas after correction for the relative sensitivity of each component to G.L.C. analysis.

Jefferies[52] had shown that a linear relationship occurred between sitosterol T.M.Si-ether and cholestane on 1 metre columns of OV101. A linear relationship was obtained on the 2 metre SE30 column used in this assay.

(1) Extraction of lipid fraction

The free sterol component was extracted from dried, powdered, tissue culture cells by petrol extraction for 24 hours in a soxhlet apparatus. Subsequent extraction with fresh petrol had shown that the cells were fully extracted after 24 hours. Examination of the crude extract showed the presence of no sapogenin which would interfere with the G.L.C. analysis, but it was necessary to isolate the sterol from fixed oil and other components which interfered with the response of the internal standard.

(2) Isolation of the free sterol

The absence of sapogenin in the extracts meant that activity II silica gel gave a satisfactory separation of the phytosterol from other extract components when used in the column chromatography system. The columns were packed, as before, with a slurry of silica gel, in hexane: ethyl acetate

9:1, to a length of 31 cm and run with the same flow rate of 1-1.5 ml/min.

The use of activity II silica gel reduced the time, and the amount of solvent, required to elute each column. The first 10 ml fraction of solvent contained fixed oil and esters, and the sterol fraction was collected in the fractions 10-40 ml.

No attempt was made to recover the column. It was found to be quicker to re-pack with fresh silica-gel. The use of fresh adsorbent overcame the problem of maintaining the activity of the column and the consequent use of 'wet' solvents.

(3) The G.L.C. determination of sitosterol as a T.M.Si-ether

The calibration was carried out by two methods. The peak areas were determined by disc integrator and by multiplying peak heights by the width of the peak at half the height, TableII.3I. A linear relationship was obtained by both methods, Fig.II.23Later it was found easier to assay the sitosterol present in an extract by the latter method. The tail of the stigmasterol peak was often found to interfere with the sitosterol peak and the estimation of the background 'count' necessary in the disc integrator method became

Table II.3I

Gas chromatographic calibration of sitosterol as T.M.Si-Ether.

Comparison of data obtained using two methods of peak evaluation

Weight sitosterol µg	Mean ratio sitosterol to cholestane	
	Disc Integrator	Peak Measurement
102.50	0.95	1.18
410.25	4.40	4.80
512.80	5.50	6.00
615.38	6.80	7.20
820.51	9.20	9.70

By Disc Integrator

Slope = .01148

Confidence Interval = 5.37 ± 0.146

or $5.37 \pm 2.72\%$

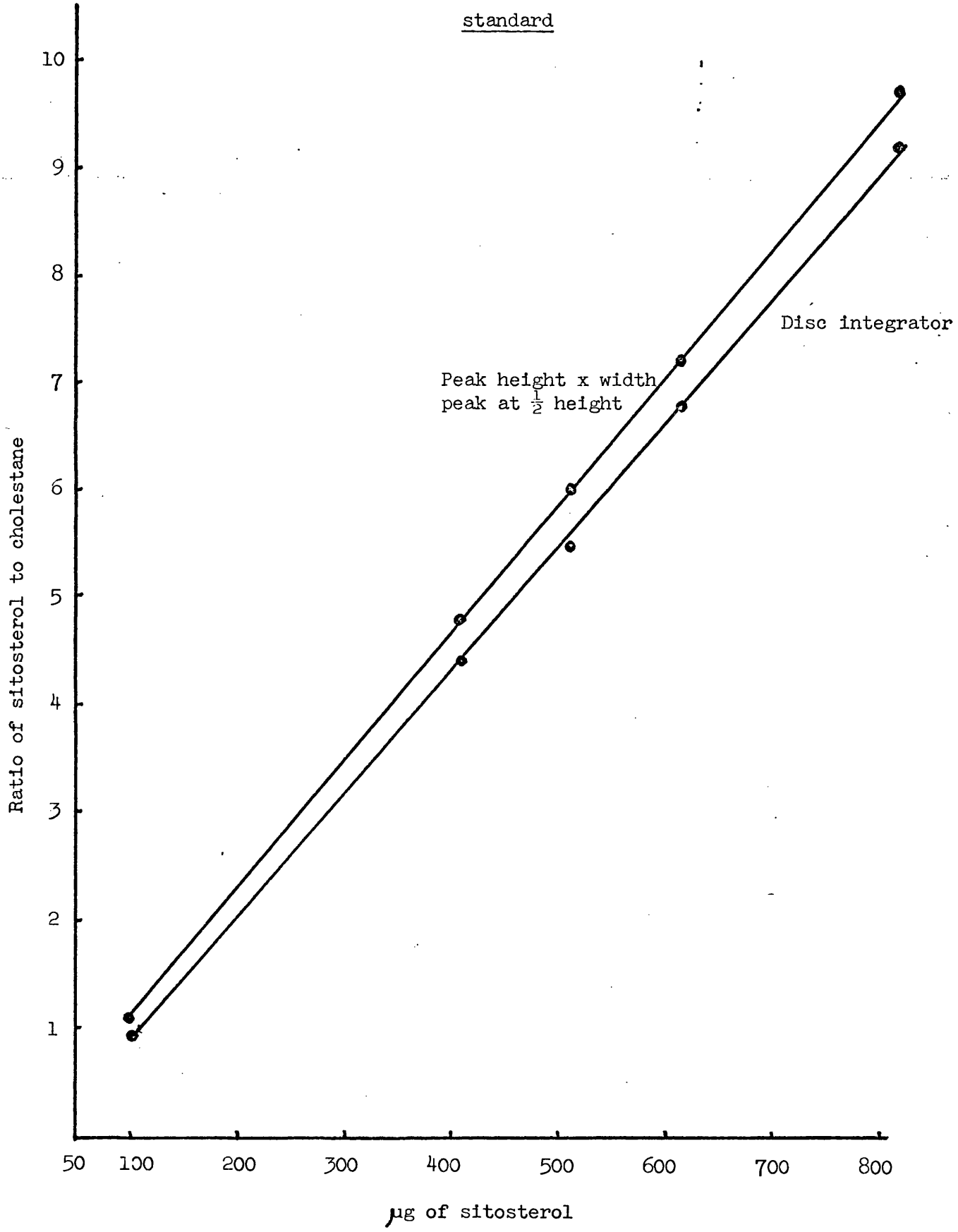
By Peak Measurement

Slope = .01184

Confidence Interval = 5.78 ± 0.149

or $= 5.78 \pm 2.58\%$

Fig II.23 Gas chromatographic calibration for sitosterol T.M.Si-ether by disc integrator and peak measurement methods with cholestane as internal standard



difficult when the stigmasterol component was large. Employment of the alternative method overcame this difficulty and more consistent results were possible.

(4) Estimation of the sterol recovery from the adsorption chromatography column

A solution of sitosterol standard containing 500 µg/ml in hexane:ethyl acetate 9:1 was prepared. To each of eight trial columns 0.5 ml was added with an Agla syringe. The columns were run and the fractions collected and prepared for G.L.C. analysis. Two G.L.C. determinations were carried out for each recovered fraction, Table II.32

The recovery obtained using only 9:1 was 92 to 102.8%, giving a mean result of 245 µg, compared with the theoretical result of 250 µg and a coefficient of variation of 3.55%.

(5) The determination of error of the sitosterol assay procedure

The overall error of the assay procedure, including petrol extraction and adsorption column chromatography separation, was tested by conducting a series of eight determinations on a standard sample of tissue. A 20 g sample of dried tissue was powdered, sieved and thoroughly mixed. Eight aliquots were weighed accurately, extracted and assayed, Table II.33. The mean value for sitosterol was 195.85 µg/g $\pm$ 11.13%. For duplicate results, as employed in the routine analysis of tissue samples, the corresponding error was calculated to be 195.85 $\pm$ 7.87%.

Table II.32

Sitosterol recovery from adsorption chromatography column

Column	Sitosterol recovered	% Recovery
1	257 µg	102.8
2	248	99.2
3	240	96.0
4	230	92.0
5	248	99.2
6	257	102.8
7	240	96.0
8	240	96.0

Mean recovery 245 µg or 98%

Table II.33

The determination of error of the sitosterol assay procedure

Sample weight	Weight of sitosterol 1 g of callus	% m.f.b.
1.3404	209 µg	.0209
1.4136	198 "	.0198
1.4448	194 "	.0194
1.4821	189 "	.0189
1.4357	188 "	.0188
1.5097	186 "	.0186
1.2996	192 "	.0192
1.3259	211 "	.0211

Coefficient of Variation = 4.84%

The percentage range of error for duplicate determination at P = 0.05 is 195.85 $\pm$ 7.87%

(6) Determination of the relative weight response of the phytosterols identified in culture extracts as T.M.Si-ethers on a 6 ft 2½% SE30 column

It was found that the major phytosterol present in extracts of tissues grown on NAA was sitosterol. Tissues grown on 2,4-D had two major peaks which were identified as sitosterol and stigmasterol. Both tissues had minor peaks corresponding to cholesterol and campesterol. The responce of the four sterols relative to the response of cholestane were determined. Solutions of pure standards of each phytosterol were prepared as T.M.Si-ethers with a known amount of cholestane as internal standard. The peak areas were measured for each by the disc integrator method, and the relative weight response calculated. The responses of the four phytosterols, relative to cholestane, were, cholesterol 0.85, campesterol 0.80, stigmasterol 0.69 and sitosterol 0.66. The relative weight response was calculated from the formula

$$\frac{\text{Sterol peak area} \times \text{wt. of cholestane}}{\text{wt. of sterol} \times \text{cholestane peak area}}$$

An estimation of the amount of each sterol present in an extract could then be made by reference to the amount of sitosterol present, after correction of the peak areas with the appropriate weight response value.

PART II CHAPTER VI

A COMPARISON OF THE SAPOGENIN AND PHYTOSTEROL CONTENT OF CALLUS TISSUES GROWN ON NAA AND 2,4-D IN CONTINUOUS LIGHT

EXAMINATION OF THE FREE STEROL AND SAPOGENIN

Tissues which had been grown for six months, on MS medium containing 10 ppm NAA and 10% v/v coconut water, were sub-cultured onto MS medium containing 2,4-D at concentrations of 0.01, 0.1 and 1.0 ppm and 10% v/v coconut water. After a further six months growth in continuous light at 25°C, all the cultures, irrespective of the auxin used, afforded, by acid hydrolysis and TLC of the extract, both phytosterol and sapogenin spots. From the size of the spots on the TLC it appeared that the sapogenin level from cultures grown with 2,4-D was lower than from the cultures grown on NAA, but no difference was apparent in the phytosterol levels. No other differences were seen from the TLC. Khanna[37] reported maximum sapogenin yield from 56 day old Fenugreek tissue cultures grown on MS medium containing 2,4-D. GLC assay of 56 day old cultures grown in the presence of the different auxins, showed differences in both the diosgenin and phytosterol content, in that tissues grown with 10 ppm NAA were found to contain approximately 0.02% m.f.b. of diosgenin/yamogenin, but those grown with the 2,4-D, contained only trace amounts of diosgenin/yamogenin, which could not be quantified, Table II.34

Tissue grown with the lowest concentration of 2,4-D (0.01 ppm) did appear to contain more sapogenin than tissues grown on the other two concentrations and an approximate

Table II.34

The diosgenin/yamogenin yield from tissue cultures grown on MS medium with NAA or 2,4-D

(Mean of two determinations)

Auxin in the medium	Diosgenin/Yamogenin % w/w m.f.b.	
2,4-D		
0.01 ppm	Trace	.006
0.10 ppm	"	Trace
1.00 ppm	"	"
NAA 10 ppm	0.0207	0.0222

estimation of the sapogenin recovered was made.

In later work published by Drain et al[38] it was reported that callus cultures grown on MS medium with 0.25 ppm, 2,4-D and 10% v/v coconut water yielded approximately 0.003% m.f.b. of diosgenin. Khanna,[37] however, using MS medium and 1 ppm 2,4-D reported a diosgenin content of 1.8% m.f.b., but did not name the cytokinin, if any, used. This concentration was greater than has been reported in extracts from whole Fenugreek seed.

The nature and amount of free phytosterol obtained from tissues grown with the different auxins also varied. The predominant free sterol from tissue grown with 10 ppm NAA was sitosterol. A smaller amount of stigmasterol and traces of campesterol and cholesterol were also detected. In contrast, from tissues grown with 2,4-D the major free sterol was either stigmasterol or sitosterol, but at all three concentrations of 2,4-D used, larger amounts of stigmasterol were detected than in NAA cultures. Cholesterol, campesterol and two unidentified components were also detected in the free sterol extracts of tissues grown with both NAA and 2,4-D. The tissue grown with 0.01 ppm 2,4-D had a comparatively higher cholesterol content than any of the other tissues, including that from material grown with NAA.

The sitosterol component of the free sterol fractions was quantitatively determined. The peak areas were calculated by both the disc integrator and peak measurement methods and the results compared.

Tables II.35 & II.36 show that the values obtained by measurement of peak areas were close to those given by the disc integrator method. Any slight overlap in the 'tails' of the stigmasterol and sitosterol were ignored in the estimation of peak area by peak measurement and by using this method an approximate value could be obtained for the minor peak, campesterol, which was only partially separated from the major stigmasterol peak.

It was found that sitosterol and stigmasterol together represented approximately 90% of the free sterol, Tables II.37 to II.40 The tissues grown on the three media with 2,4-D afforded more free sterol than the tissue grown on 10 ppm NAA. The ratio of stigmasterol to sitosterol for tissue grown with 10 ppm NAA was 0.38:1, whereas from tissue grown with 2,4-D the ratio was 0.86:1 (0.01 ppm), 1.57:1 (0.1 ppm) and 1:1 (1 ppm) where the concentrations of auxin are given in brackets. The high ratio of stigmasterol to sitosterol observed in tissue grown on medium containing 0.1 ppm 2,4-D was accompanied by a fall in the amount of sitosterol, so that the total major free sterol yield was similar to that from tissue grown on 0.01 ppm 2,4-D.

Since the tissues grown on media containing 2,4-D and NAA were both derived from the same stock culture (grown on MS medium with 10 ppm NAA and 10% v/v coconut water) it was concluded that the biosynthesis of sterols was affected

Table II.35

A comparison of the methods for determining peak areas in the assay of sitosterol present in free sterol extracts from dried callus.

Disc Integrator Method

Auxin in medium	sample weight g.	Mean ratio relative to cholestane	Wt assayed in µg	Sitosterol % m.f.b.
10 ppm NAA	1.6298	2.60	247	.0151
	1.9944	2.97	277	.0138
0.01 ppm 2,4-D	1.4660	5.52	507	.0345
	1.6440	5.98	548	.0333
0.1 ppm 2,4-D	1.7910	5.10	470	.0262
	1.6990	5.16	473	.0279
1 ppm 2,4-D	1.4641	6.07	550	.0375
	1.5672	7.16	650	.0414

Table II.36

A comparison of the methods for determining peak areas in the assay of sitosterol present in free sterol extracts from dried callus

Peak Measurement Method

Auxin in medium	Sample weight g.	Mean ratio relative to cholestane	Wt assayed in µg	Sitosterol % m.f.b.
10 ppm NAA	1.6298	3.01	255	.0156
	1.9944	3.16	270	.0135
0.01 ppm 2,4-D	1.4660	6.46	550	.0375
	1.6440	6.80	5.78	.0351
0.1 ppm 2,4-D	1.7910	5.53	472	.0263
	1.6990	6.00	510	.0300
1 ppm 2,4-D	1.4641	6.46	550	.0375
	1.5672	7.74	660	.0421

Table II.37

Estimation of sitosterol + stigmasterol in the free sterol extract

(Determinations in duplicate)

Auxin in medium	Mean ratios relative to cholestane				Stigmasterol/ sitosterol	Stigmasterol % m.f.b.	Major sterols % m.f.b.
	Sit. *	Stig. *	Sit. **	Stig. **			⁎
10 ppm NAA	3.01 3.16	1.15 1.26	4.43 4.78	1.66 1.82	0.37 0.38	.0057 .0051	.0214 .0186
.01 ppm 2,4-D	6.46 6.80	5.61 6.33	9.79 10.30	8.13 9.17	0.83 0.89	.0311 .0312	.0686 .0664
0.1 ppm 2,4-D	5.53 6.00	8.90 10.05	8.38 9.10	12.90 14.56	1.54 1.60	.0406 .0448	.0669 .0728
1.0 ppm 2,4-D	6.46 7.74	6.96 7.77	9.79 11.73	10.08 11.26	1.03 0.96	.0386 .0404	.0762 .0825

* Uncorrected for relative weight response

** Corrected for relative weight response

⁎ Sitosterol + stigmasterol

Table II.38

Estimation of the minor free sterols : Campesterol

(Determinations in duplicate)

Auxin used in medium	Ratio relative to cholestane	Ratio corrected for sensitivity	Campesterol/ sitosterol	Campesterol % m.f.b.
10 ppm NAA	.41	.51	.11	.0017
	.47	.58	.12	.0016
0.01 ppm 2,4-D	.58	.73	.07	.0026
	.47	.59	.06	.0021
0.1 ppm 2,4-D	.70	.87	.10	.0026
	.65	.81	.09	.0027
1.0 ppm 2,4-D	.38	.50	.05	.0018
	.68	.85	.07	.0029

Table II.39

Estimation of the minor free sterols : Cholesterol

Auxin used in medium	Ratio relative to cholestane	Ratio corrected for sensitivity	Cholesterol/ sitosterol	% m.f.b. of cholesterol
NAA 10 ppm	.25 .30	.29 .35	.06 .07	.001 .001
2,4-D 0.01 ppm	.87 .89	1.05 1.02	.11 .09	.004 .003
2,4-D 0.1 ppm	.57 .62	.68 .74	.08 .08	.002 .002
2,4-D 1.0 ppm	.21 .23	.25 .27	.02 .02	.001 .001

Table II.40

Estimation of the composition of the free sterol extract : Summary

(Mean of duplicate results)

	% m.f.b.				
Auxin in the medium	Sitosterol	Stigmasterol	Campesterol	Cholesterol	Total
NAA 10 ppm	.0146	.0055	.0017	.0009	.0227
2,4-D .01 ppm	.0362	.0312	.0024	.0036	.0734
2,4-D 0.1 ppm	.0282	.0427	.0027	.0022	.0758
2,4-D 1.0 ppm	.0398	.0395	.0024	.0008	.0825

either by the type, or the concentration of auxin in the medium. A medium was prepared containing 1 ppm NAA and innoculated with tissue grown on the stock culture medium containing 10 ppm NAA. It was grown alongside the cultures previously examined, for four months, under the same conditions, with subculturing after one and two months. The free sterol and diosgenin/yamogenin content of the tissues was re-examined.

Diosgenin/yamogenin was again detected in only trace amounts in tissue grown with 2,4-D and tissues grown with both concentrations of NAA continued to synthesise diosgenin/yamogenin at concentrations of about 0.02% m.f.b. The free sterol pattern remained essentially the same, table II.4/42, figs II.24&25 with relatively more stigmasterol present in tissue grown on 2,4-D. Tissue grown on 1 ppm NAA retained the sterol pattern observed in the original culture on 10 ppm NAA. The amounts of free sterol extracted from the cultures were different to the first experiments.

The ratio of stigmasterol to sitosterol of tissue grown on 1 ppm and 10 ppm NAA was 0.32:1. In all the cultures grown on media containing 2,4-D the ratio of the two sterols was approximately 1:1. Tissues grown on 10 ppm and 1 ppm NAA yielded twice the amount of sterol extracted previously from NAA grown tissue. Extracts from tissues grown on 0.01 ppm and 1.0 ppm 2,4-D contained approximately 50% less free sterol whilst tissue grown on 0.1 ppm 2,4-D yielded twice as much as previously. It was concluded that sterol and sapogenin biosynthesis in Fenugreek tissue cultures was affected by the

Table II.4I

The determination of the sitosterol present in the free sterol extracts of dried callus

(Second experiment)

Auxin in the medium	Sample wt in g	Mean ratio relative to cholestane	Wt assayed in µg	Sitosterol % m.f.b.
1 ppm NAA	1.9833	7.82	665	.0335
	1.8818	8.31	707	.0375
10 ppm NAA	1.9860	8.25	700	.0352
	1.7846	8.05	685	.0384
.01 ppm 2,4-D	2.1230	5.89	500	.0235
	2.2320	6.82	580	.0250
.1 ppm 2,4-D	1.8531	9.81	835	.0451
	1.9817	10.10	870	.0439
1.0 ppm 2,4-D	2.1531	5.69	482	.0223
	1.9979	5.21	442	.0221

Table II.42

Estimation of sitosterol + stigmasterol in the free sterol extracts

(Determination in duplicate)

(Second experiment)

Auxin in medium	Mean ratios relative to cholestane				Stigmasterol/ sitosterol	Stigmasterol % m.f.b.	Major sterols % m.f.b.
	Sit. *	Stig. *	Sit. **	Stig. **			⁂
1 ppm NAA	7.82	2.58	11.85	3.74	.32	.0105	.0441
	8.31	2.82	12.59	4.10	.32	.0122	.0498
10 ppm NAA	8.25	2.73	12.50	3.96	.32	.0111	.0464
	8.05	2.58	12.19	3.74	.31	.0117	.0501
.01 ppm 2,4-D	5.89	6.11	8.92	8.86	.99	.0233	.0469
	6.82	6.99	10.33	10.13	.98	.0254	.0514
.1 ppm 2,4-D	9.81	9.54	14.86	13.82	.93	.0418	.0870
	10.10	9.83	15.30	14.25	.93	.0408	.0847
1.0 ppm 2,4-D	5.69	4.64	8.61	6.72	.78	.0174	.0398
	5.21	4.75	7.90	6.88	.87	.0192	.0414

* Uncorrected for relative weight response

** Corrected for relative weight response

⁂ Sitosterol + stigmasterol

Fig. II.24

The gas chromatographic analysis of T.M.Si-ethers of the free sterols of Fenugreek tissue cultures grown with NAA

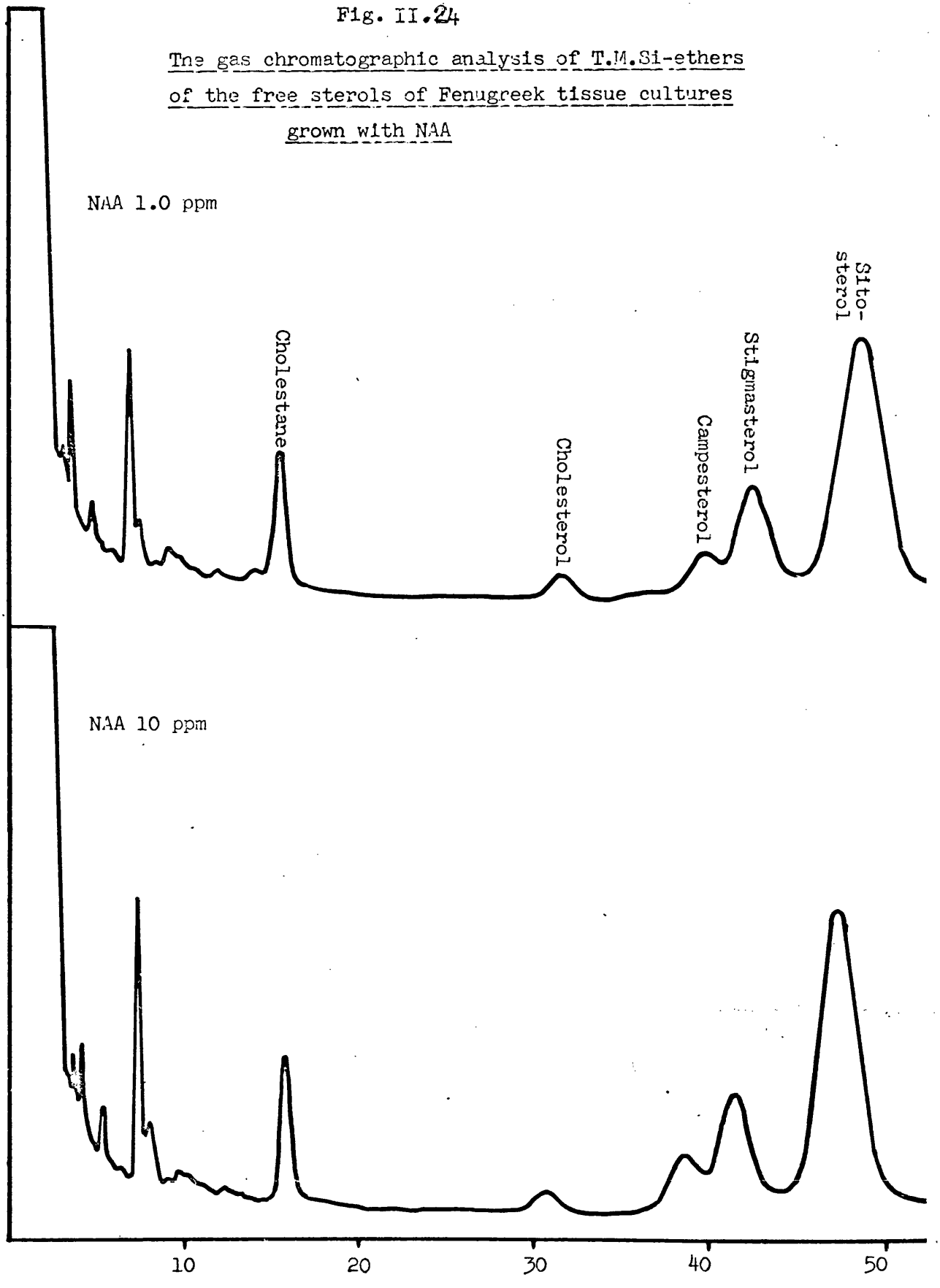
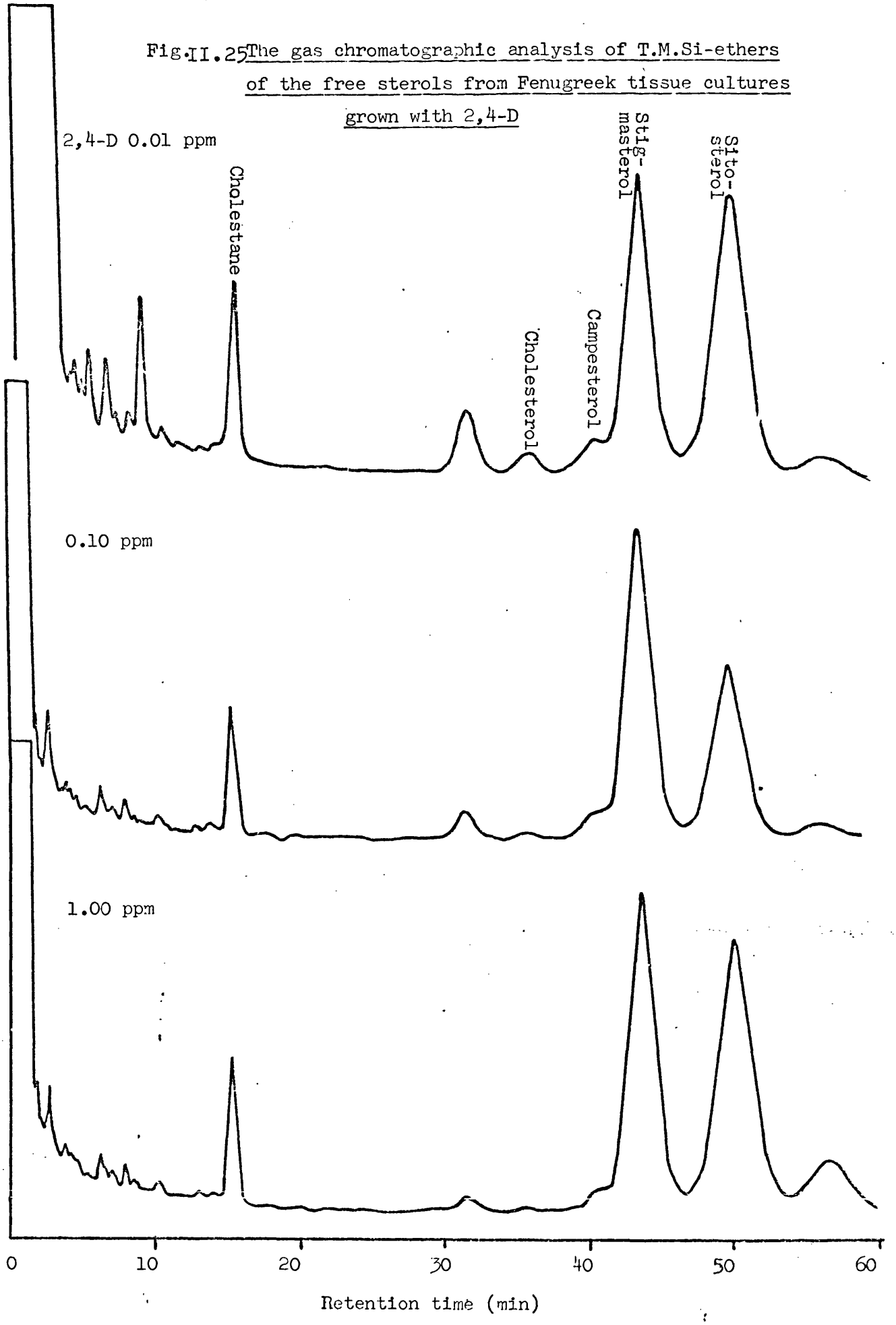


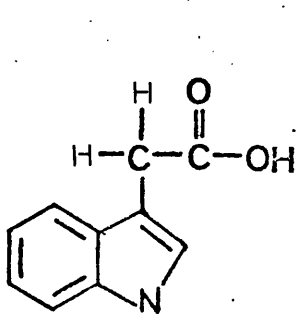
Fig.II.25 The gas chromatographic analysis of T.M.Si-ethers of the free sterols from Fenugreek tissue cultures grown with 2,4-D



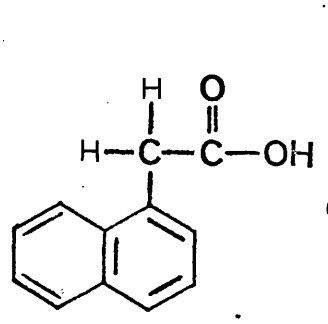
auxin used as a growth regulator. Similar effects have been observed in tissue cultures of Solanum xanthocarpum.[33] The use of IAA as the growth regulator instead of 2,4-D was found to result in a two-fold increase in diosgenin, but a reduction in sitosterol content. In Fenugreek cultures the use of 2,4-D instead of NAA also reduced the diosgenin content.

The chemical structures of IAA and NAA are similar with NAA possessing a 6 membered, benzene, ring in place of the pyrrole ring of IAA. Van Overbeek[79] envisaged the primary mode of auxin action as involving physico-chemical bonding to the lypoprotein membrane rather than on a key enzyme system. Specificity would depend on the fit of the ring structure into its appropriate hole in the cyto-skeleton and projection of the carboxyl groups as a net-work above the membrane surface.

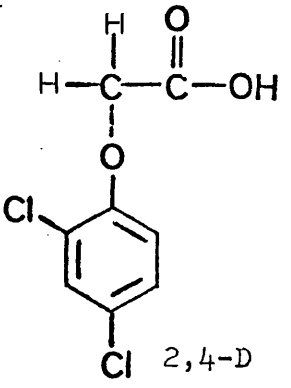
Freed et al[79] proposed that the predominant mechanism of action for the chlorophenoxyacetic acids was adsorption on a protein surface with modification of the protein structure in such a way as to change its enzymatic activity. The fall in sapogenin content of Fenugreek tissue cultures in the presence of 2,4-D was, therefore, possibly caused by the specific binding of the auxin to a lypoprotein membrane, or/a protein enzyme in the biosynthetic pathway of sapogenin, with consequent modification of its activity. Similarly the change in free sterol content of the tissue could have been caused by enzyme, or membrane, modification.



I.A.A.



N.A.A.



2,4-D

Heftmann [80] concluded that the role of sterols in micro-organisms, plant and animals is essentially the same. Sterols, or their immediate precursors, are the starting material for all subsequent steroids and one of the functions of sterols is to provide an inert stockpile of precursors that can readily be converted to biologically active components.

Sterols have been implicated in the structure of cellular membranes.[81] The fungi Pythium and Phytophthera do not synthesise, or apparently require, sterols for membrane activity or vegetative growth, but Seitsma and Haskins[82] found that cholesterol was taken up by these organisms and incorporated, unchanged, into the cell membrane. The temperature tolerance of the organisms was increased and it was concluded that cholesterol reduced the cell wall permeability. The polyene anti-biotics are active against yeasts and moulds. The selective toxicity of these compounds, with relation to the sterol composition of the organisms affected, has been studied.[83] The conclusion has been drawn that they interact with sterols in the cell membrane and permit the leakage of material from the fungal cell. The antagonism between

sterols and polyene antibiotics permits the reversal by cholesterol of the antibiotic-induced growth inhibition.[80] Evans[84] demonstrated the rapid incorporation of $^{14}CO_2$ into the mature leaf sterols of Digitalis purpurea. He concluded that a rapid turnover of steroid material in the cell and organelle membranes took place and that sterols had a dynamic role in the functions of the lipo-protein membrane. Olsen[39] is of the view that the sterols control the permeability of the membrane by causing the phospho-lipid molecules to pack together.

Heftmann[80] also suggested that steroids play a hormonal role in all cells, similar to that observed in animals, and this is supported by work on both plants and fungi. Animal steroidal sex hormones have been shown to affect flowering in plants. Love and Love[85] reported that the application of androgens or oestrogens to the stem of Melandrium divecum resulted in either male, or female, flower development. The biosynthetic inhibitor, SK and F 7997-A3, which blocks lanosterol sterol/conversion to zymosterol in animal systems, was shown to suppress floral induction in Xanthium.[86] The similarity in metabolism of fungi and plants has been demonstrated by the synthesis of auxins, gibberellins and cytokinins by fungi. The fungus Fusarium synthesises gibberellin[87] and Taphrina rudebeckii synthesises IAA. Rypacek and Sladky[88] bio-assayed growth regulators extracted from the fungus Lentinum tigrinus and found auxins and gibberellins present in vegetative mycelium and cytokinin in the primordia of caps. Steroids have been shown to affect both vegetative and sexual growth in fungi. Elliot, Hendrie et al[90] have shown that oospore formation takes place after three days of contact

with the appropriate steroid.

On work with Pythium, Sietsma and Haskins[83] added IAA to cultures and obtained increased activity of the two enzymes responsible for the synthesis of the two major cell wall components. In the presence of cholesterol the branching effect on hyphae, which occurred as a result of IAA stimulation, was removed. The sterol extract of Pisum sativum was found to have a stimulating effect on both the vegetative growth and sporulation of Phytophthera cinnamoni. Similar effects were achieved when cholesterol, stigmasterol and sitosterol were added individually. The sterols present in plants may, therefore, play an active part in the regulation of all growth and metabolism and a close relationship between their action and that of the recognised growth regulators may exist. Hendrix and Guttman found that cholesterol induced reproduction in Pythium periplocum was inhibited by the steroid oestradiol. In Pisum sativum [91] seedlings, the addition of oestradiol resulted in increased auxin synthesis. Kopcewicz postulated that oestrogens influenced both the auxins and gibberellins in plant tissues, in investigations into the control of growth and development through the combined action of several regulating substances.

It is therefore possible that the change in sterol pattern observed in Fenugreek tissue cultures as a response to different growth regulators may be part of a hormonal 'feed back' mechanism. The ratio of the different sterols could be indicative of a very fine control mechanism of the plant metabolism. Some changes observed in the sterol composition of developing plants substantiate this hypothesis. Bush and

Grunwald[92] have shown that in germinating seedlings of Nicotiana tabacum stigmasterol was initially low, but increased after three to four days. The free cholesterol and campesterol content doubled in this time. Ingram et al[93] found a significant rise in the cholesterol fraction of oat seedlings within the first two days of germination. The use of SKF 7997-A_3 in cultures of excised tomato roots resulted in an inhibition of both the steroidal alkaloid synthesis and growth of the cultures.[94] Roddick and Butcher[94] suggest that the step inhibited may be common to certain growth processes and tomatine biosynthesis and that the compound concerned is a steroid, after zymosterol, in the biosynthetic pathway, It has, however, been shown[95] that SK 7997-A_3 and similar substances are likely to affect metabolic pathways in fungi (Phytophthora cactorum) that do not synthesise sterols; therefore care has to be taken in conclusions derived from the use of these substances.

The relative amounts of cholesterol, campesterol, sitosterol and stigmasterol in Fenugreek cells may, therefore, be related to the maturity of the cells. The sterol pattern of tissue grown on NAA resembles that of the extract from the original mature seed in which the stigmasterol content is very low.

EXAMINATION OF THE BOUND STEROLS

Evans [96] concluded that in Digitalis purpurea the biosynthesis of sterols occurred in the free form and that glucoside or ester formation occurred when biosynthesis was complete. Free sterols were implicated in membrane structures and sterol esters were thought to be involved in the intracellular transport of sterol.[97] Evans [96] suggested that in the intact plant the glycoside sterols were involved in intercellular transportation from areas of synthesis to areas of growth where sterols were required for membrane expansion. The sterol recovered from the glycoside and ester fractions of the tissues grown on the media used in the previous experiment were examined.

(1) Examination of the glycoside sterol fractions

Powdered samples of dried tissue weighing approximately 1.5 g were extracted with light petroleum for 24 hours in a soxhlet apparatus to remove fixed oil, steryl esters and free sterol. The tissue was dried, in a vacuum oven, to remove the petrol and hydrolysed with 2N hydrochloric acid for 2 hours. A crude extract was obtained by extraction of the dried, neutralised residue with chloroform in a soxhlet apparatus for 24 hours. The sterol was isolated by

adsorption column chromatography, using the method employed in the assay of sapogenin. After conversion to T.M.Si-ethers the sterol fractions were analysed by G.L.C.

The sterol fractions exhibited a similar compositon to that observed in free sterol fractions. In tissues grown on media containing NAA the major sterol component was sitosterol. Stigmasterol was the major sterol component of tissues grown on 2,4-D. The experiment provided only a qualitative examination of the sterols because the losses incurred in the extraction procedure were not known. The ratios of the two major sterols present in the extracts is given in Table. II.43

(2) Examination of the sterol esters

The free sterol extracted in the lipid fraction in the previous experiment was isolated from the sterol ester and fixed oil by adsorption column chromatography. A 31 cm x 0.5 cm column was packed with activity II silica gel in a slurry of hexane:ethyl acetate 9:1. The lipid fraction was loaded with 2 ml of the same solvent and eluted with a further 9 ml to remove the fixed oil and ester fraction, leaving the free sterol on the column.

The oil and ester fraction was evaporated to dryness and refluxed with 0.5N potassium hydroxide in alcohol for 1 hour. The unsaponifiable matter was shaken out with diethyl ether. After the ether fraction had been washed with water the solvent was removed and the residue dried. The adsorption chromatography column procedure was repeated to isolate the sterol, a T.M.Si-ether was prepared and G.L.C. analysis performed. In the cultures examined the ester fraction was very small and insufficient sterol was recovered for analysis. Further work is required to develop reliable techniques for monitoring this fraction.

Table II.43

The ratio of stigmasterol to sitosterol in sterol glycoside extracts of tissues grown on MS medium with 2,4-D and NAA

Trial	Ratio Stigmasterol:sitosterol
1 ppm NAA	0.30:1 0.31:1
10 ppm NAA	0.43:1 0.50:1
.01 ppm 2,4-D	1.21:1 1.22:1
0.1 ppm 2,4-D	1.34:1 1.32:1
1.0 ppm 2,4-D	1.10:1 1.09:1

ALTERATION OF THE CONDITIONS EMPLOYED FOR TISSUE CULTURE GROWTH

THE EFFECT OF DIFFERENT LIGHT CONDITIONS ON THE DIOSGENIN/YAMOGENIN YIELD OF FENUGREEK TISSUE CULTURES

The effect of different light conditions on the biosynthetic processes of some tissue cultures has been studied. Sunderland [I] observed changes in the cell protein of tissues of Oxalis dispar, transferred from dark to light conditions, when chloroplast formation took place. The cell protein content of green tissues was slightly higher and the growth rate lower. He suggested that re-orientation of protein synthesis occurs when tissues are subjected to light.

Light conditions were found to be critical for the biosynthesis of anthocyanins [98] by tissue cultures of five different plants. Cultures which had been maintained in 12 hour photoperiods at 2500 lux, only produced anthocyanins when transferred to continuous light at an intensity of 7500 lux. Work on cultures of Kalanchoi crenata [99] has shown an increase in mevalonate actuating enzymes which was thought to be directly connected with chloroplast formation. The introduction of $2C^{14}$ mevalonic acid into cultures resulted in the formation of labelled mevalonate-5-phosphate, mevalonate-5-pyrophosphate and isopentylpyrophosphate in green cultures. The compounds were not detected in Kalanchoi cultures grown in the dark after a similar growth period. Thomas [99] suggested that, as the chloroplasts developed, more substrate from fixation of carbon dioxide became available for incorporation into the terpenoid pathway via MVA and a resultant induced activation of pre-existing enzyme or 'de novo' enzyme synthesis occurred.

Previous workers investigating the synthesis of sapogenins

by tissue culture systems have given few details of the light conditions employed, or even considered the effect of light on sapogenin synthesis. Kaul and Staba[46 27] grew tissue and suspension cultures of Dioscorea deltoidea under 'ordinary room light conditions'. Tomita et al[100] made no mention of light conditions in their study of diosgenin from Dioscorea tokoro cultures and Heble et al[101] specify no conditions in their examination of steroidal alkaloids and diosgenin from Solanum xanthocarpum. Brain et al[38] gave no conditions in their work on the steroidal constituents of Fenugreek suspension cultures. Khanna et al[36] used 'normal room light' in early work on Fenugreek tissue cultures. In later investigations of the steroidal constituents of Fenugreek cultures, Khanna and Jain[37] specified 18 hours daily illumination, at 1500-1800 lux, from incandescent lamps and the resultant cultures yielded large amounts of sapogenin.

The sapogenin content of Fenugreek tissue cultures which had been maintained in continuous light, or dark, conditions for 12 months, was determined to see if the conditions affected the sapogenin biosynthesis. Cultures which had been maintained by subculturing onto fresh medium at 30 day intervals were inoculated onto 100 ml aliquots of MS medium, containing 10 ppm NAA and 10% v/v coconut water and grown for a period of 56 days. The cultures were harvested, oven dried at 60°C and assayed for diosgenin/yamogenin. Sufficient fresh tissue was retained to inoculate fresh medium and the experiment was repeated for two subsequent 56 day growth periods.

The diosgenin content of tissue grown in the dark was

lower in the first determination, but not significantly different from light grown tissue in the two subsequent determinations, see Table II.44. The diosgenin/yamogenin yield of both sets of cultures was higher at 0.02% m,f.b. than that from callus cultures grown on the same medium, but with 2,4-D, by Brain et al[38] (.003%), when no light conditions were stated. Khanna et al[37] reported a total sapogenin from Fenugreek callus culture of 4.19% and a diosgenin level of 1.8% m.f.b. with 18 hour photoperiods. Another experiment was carried out to investigate the effect of cyclic light conditions on sapogenin yield.

Cultures were grown for 56 days under fluorescent lights (Ediswan warm white) at a light intensity of 1500-1800 lux, with a light period of 18 hours per day at $25^{\circ} \pm 1^{\circ}$. The diosgenin/yamogenin content was compared with cultures grown in total darkness for the same period of time and at the same temperature. The tissues were subcultured at the time of harvesting and grown for a second 56 day period under the same conditions.

The results show a slight, but not significant, difference in the sapogenin yield, see TableII.45. It was concluded that the light conditions, as employed by Khanna,[37] were not a critical factor affecting sapogenin biosynthesis in our experiments.

Table II.44

A comparison of the diosgenin/yamogenin yield from tissues grown in continuous light and continuous dark for a period of 56 days

Conditions of growth	Sample wt. g	Mean ratio relative to cholestane	Wt. in μg of D + Y	% w/w of m.f.b.	Mean % w/w of m.f.b.
Light	2.5402 1.8850	6.50 5.08	528 420	.0207 .0222	.0215
Dark	2.7845 2.7681	3.18 3.15	270 268	.0096 .0096	.0097
Light	2.1796 2.1017	4.95 3.62	410 305	.0188 .0145	.0167
Dark	2.6286 2.8345	4.60 5.65	380 465	.0144 .0164	.0154
Light	2.2857 2.4917	6.44 6.89	526 560	.0230 .0224	.0227
Dark	2.7936 2.2087	8.36 6.25	675 510	.0241 .0230	.0236

Table II.45

A comparison of the diosgenin/yamogenin yield from tissues grown in 18 hour photoperiods and continuous darkness for 56 days

Conditions of growth	Sample wt.	Mean ratio relative to cholestane	Wt. in µg of D + Y	% w/w of m.f.b.	Mean % w/w of m.f.b.
Alt. light and dark	2.5984 2.6621	7.15 6.50	577 530	.0222 .0199	.0210
Dark	2.4040 2.4768	5.41 5.39	445 443	.0185 .0180	.0182
Alt. light and dark	2.1457 2.2838	5.18 5.26	420 425	.0195 .0186	.0191
Dark	2.4314 2.6810	4.45 5.61	370 460	.0152 .0171	.0162

THE GROWTH OF FENUGREEK TISSUE CULTURES ON MS MEDIUM CONTAINING 4-HYDROXYISOLEUCINE

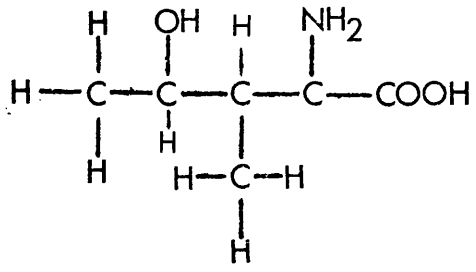
Plants have no essential amino acids and are able to synthesise their requirements from the three amino acids, glutamic acid, aspartic acid and alanine, which can in turn be synthesised from nitrogen in the form NH_4^+. The biosynthetic capabilities of tissue cultures cannot be presumed and some cultures require amino acid supplements to maintain growth. Glycine is commonly added to media and natural complex supplements, such as casein hydrolysate and yeast extract, have been used to provide amino acid mixtures. The amino acids of yeast extract were studied by Sandstedt and Skoog[102] They found that any of the amino acids in the mixture, with the exception of isoleucine, could be omitted from their medium without loss of growth stimulation of tobacco callus. Isoleucine was found to antagonise growth inhibition by valine in their cultures. For the growth of cell suspension cultures Torrey and Reinert[9] devised a medium containing 18 amino acids including DL isoleucine and L-leucine.

Furuhashi and Yatazawa[103] showed that in some circumstances the presence of isoleucine in a medium could adversely affect the growth of rice cultures. It was found that poor growth, caused by the absence of methionine, occurred only in the presence of threonine and lysine, possibly because the biosynthesis of methionine is competitively inhibited by these two amino acids. Growth inhibition decreased on isoleucine

deficient medium on which the threonine content of the tissue also decreased. It was postulated that isoleucine inhibited the decomposition of threonine precursor and hence inhibited growth.

Fowden, Pratt and Smith [104] have carried out an examination of the amino acids of Fenugreek seed and isolated 4-hydroxyisoleucine as the principal unbound amino acid. This amino acid has not previously been reported in higher plants but in Fenugreek it represented 30-50% of the total free amino acid complex of the dry seed examined. Fowden et al demonstrated that its precursor was isoleucine by supplying germinating Fenugreek seedlings with (U-C^{14})L-isoleucine, in water, for an initial 24 hour incubation period. After a further 24 hours growth, 0.5% of the radioactivity supplied was present in 4-hydroxyisoleucine and incorporation reached 1% after five days.

The structure of the compound was defined by NMR and found to be as shown below.



2 amino, 3 methyl, 4 hydroxy, pentanoic acid

The two isomers (2S, 3R, 4R) and (2R, 3R, 4R) occur in Fenugreek seed and the 2S isomer is the major component.

Abul-Futuh isolated and purified 4-hydroxyisoleucine from Fenugreek seed in our laboratories, using a modification of the method of Fowden et al and an experiment was performed to monitor the growth of tissue cultures in the presence of this hydroxyamino acid.

Supniewska and Dohnal[105] analysed the free amino acids, free sugars and steroids of Solanum laciniatum tissue cultures. The amounts of amino acids present were, with two exceptions, smaller than in the original plant. The variety of amino acids detected in the tissue cultures was greater than that of the original plant. No steroidal alkaloids were detected in the tissue cultures but some of the steroids isolated from cultures were not synthesised in the plant. They concluded that the different amino acid and sugar metabolism resulted in changes in the steroid biosynthesis of 'in vitro' cultures. The sapogenins and sterols extracted from cultures grown with 4-hydroxyisoleucine were, therefore, examined. The diosgenin/yamogenin contents were assayed and the free plus aglycone sterol extracted and isolated from the hydrolysed tissue was examined. A quarter of the sterol fraction recovered from each sample was analysed by GLC and the amounts of the major sterols, sitosterol and stigmasterol, present in each callus estimated and compared.

Cultures were initially grown on the stock culture MS medium, containing 10 ppm NAA and 10% v/v coconut water, and 0, 1, 10 and 100 ppm of 4-hydroxyisoleucine. Sub-cultures were grown for 56 days in 18 hour photoperiods of

warm white fluorescent light at 25°. The diosgenin/yamogenin recovered from the four cultures was assayed and a small increase in the yield from tissue grown with 10 ppm 4-hydroxy-isoleucine was observed, Table II.46. The estimated sitosterol yield from the hydrolysed callus of the four cultures showed little variation, but the amount of stigmasterol, relative to sitosterol, extracted from tissues grown with 4-hydroxyiso-leucine was smaller than that from the control culture. The ratios of stigmasterol to sitosterol for callus grown on the three concentrations of 4-hydroxyisoleucine were 0.29:1, 0.31:1 and 0.32:1 compared with 0.39:1 for the control, Tables II.47 & II.48.

A second experiment was performed with a higher range of 4-hydroxyisoleucine concentrations and the growth of the cultures examined quantitatively. Cultures were grown on the same medium, but with 4-hydroxyisoleucine concentration of 0, 10, 100 and 1000 ppm. All the cultures grew well and no phytotoxicity was observed at the higher concentrations. No significant difference in fresh weight increase was observed and the texture and colour of the tissue produced on the three trial media were the same as the tissue grown on the control. Table II.49.

The diosgenin/yamogenin content of all the cultures grown in the second experiment was much lower than those in the first trial, but the yield from the three trial cultures was slightly higher than from the control, Table II.50. The tissue grown in this experiment was taken from the same stock culture that was used in the previous experiment and there was no apparent reason for the lower sapogenin yield.

The major sterols from the hydrolysed tissue were esti-mated and a decrease in the free, plus aglycone, sitosterol and

stigmasterol content was apparent in all the cultures. The ratio of stigmasterol:sitosterol was calculated and was again lower for the cultures grown in the presence of 4-hydroxyisoleucine than for the control. The estimated sitosterol content showed little variation for the four media. Tables II.51 , II.52.

The only difference detected in the sterol metabolism of Fenugreek tissue cultures grown on MS medium in the presence of 4-hydroxyisoleucine was a change in the relative amounts of sitosterol and stigmasterol isolated from the free plus glycoside bound sterol fractions. The yield of diosgenin/yamogenin was slightly greater from some trial cultures and the growth rate of the cultures was unaffected. The estimated amount of sitosterol in the trial cultures was the same as that of the control culture, indicating that less stigmasterol was present in the free plus aglycone sterol fraction of tissue grown with 4-hydroxyisoleucine.

Table II.46

Diosgenin/yamogenin yield from tissue grown on MS medium and 4-hydroxyisoleucine

4-Hydroxiyisoleucine in the medium	Sample wt. in g	Mean ratio relative to cholestane	Wt. in µg of D + Y	Diosgenin/ yamogenin % w/w mfb	Mean % w/w m.f.b.
Control	2.5740	10.75	862.0	.0335	.0320
0 ppm	2.3541	8.95	720.0	.0305	
	2.6763	10.95	880.0	.0328	.0312
1 ppm	2.4651	9.07	730.0	.0296	
	2.5552	12.45	995.0	.0389	.0389
10 ppm	2.6450		-	-	
	2.5674	9.43	760.0	.0296	.0280
100 ppm	2.6156	8.57	692.0	.0264	

Table II.47

Estimation of the sitosterol extracted from hydrolysed tissue grown in the presence of 4-hydroxyisoleucine

4-Hydroxyisoleucine in medium TRIAL		Wt.of sample in g.	Mean ratio relative to cholestane	Wt.assayed in µg	Total wt. in µg	Sitosterol % w/w m.f.b.
Control	1	2.5740	6.80	578	2312	0.0898
0 ppm	2	2.3541	7.01	598	2392	0.1016
		2.6763	6.89	588	2352	0.0879
1 ppm		2.4651	6.20	528	2112	0.0856
		2.5552	6.36	543	2172	0.0850
10 ppm		2.6450	-	-	-	-
		2.5674	7.18	610	2440	0.0950
100 ppm		2.6156	-	-	-	-

Table II.48

Estimation of sitosterol + stigmasterol extracted from hydrolysed tissue grown in the presence of 4-hydroxyisoleucine

4-Hydroxyisoleucine in the medium	Ratios relative to cholestane				Stigmasterol/ sitosterol	Stigmasterol % w/w m.f.b.	Major sterols % w/w m.f.b. ж
	Sit.*	Stig.*	Sit.**	Stig.**			
Control	6.80	2.60	9.85	3.94	0.40	.0359	0.1257
0 ppm	7.01	2.61	10.15	3.96	0.39	.0396	0.1412
	6.89	1.96	10.44	2.84	0.27	.0237	0.1116
1 ppm	6.20	1.86	8.98	2.87	0.32	.0274	0.1130
	6.36	1.91	9.22	2.89	0.31	.02635	0.1113
10 ppm							
	7.18	2.21	10.40	3.35	0.32	.0306	0.1255
100 ppm							

* Uncorrected for relative weight response

** Corrected for relative weight response

ж Sitosterol + stigmasterol

Table II.49

Growth response to 4-hydroxyisoleucine in the media at different concentrations

Control	4-Hydroxyisoleucine		
	10 ppm	100 ppm	1000 ppm
26.6	31.3	25.1	35.6
26.6	58.8	18.5	26.4
44.9	20.4	33.1	25.0
23.4	33.5	48.3	46.0
37.4	34.1	12.0	33.4
44.0	39.2	67.2	28.7
24.0	15.8	37.3	21.6
33.2	40.9	14.1	22.7
43.8	27.3	43.5	23.4
56.6	24.4	61.4	30.7
47.9	24.9	32.3	22.6
35.1	21.9	18.37	43.5
35.8	17.1	28.9	29.9
43.7	36.4	49.8	46.9
40.9	19.3	31.6	19.2
37.59	29.63	34.76	30.37

$F = 1.44$

From Tables

$F = 2.84$ at $P = .05$

$\therefore$ not significant

Table II.50

Diosgenin/yamogenin yield from tissue grown on MS medium and 4-hydroxyisoleucine (Second experiment)

4-Hydroxyisoleucine in the medium	Sample wt. in g	Mean ratio relative to cholestane	Wt. in µg D + Y	Diosgenin/ yamogenin % w/w mfb	Mean % w/w m.f.b.
Control	2.0818	2.85	233	.0112	.0115
0 ppm	1.8491	2.54	220	.0119	
	1.9785	3.58	303	.0153	.0155
10 ppm	1.7800	3.30	280	.0157	
	1.4480	2.44	215	.0148	.0145
100 ppm	1.7531	2.92	250	.0142	
	2.0818	3.01	307	.0148	.0151
1000 ppm	1.9255	3.52	298	.0154	

Table II.5I

Estimation of the sitosterol extracted from hydrolysed tissue grown in the presence of 4-hydroxyisoleucine (Second experiment)

4-Hydroxyisoleucine in the medium	Wt. of sample in g	Mean ratio relative to cholestane	Wt. assayed in µg	% w/w sitosterol m.f.b.
Control	2.0818	3.82	325	.0624
0 ppm	1.8491	3.22	303	.0655
	1.9785	4.16	355	.0717
10 ppm	1.7800	3.36	311	.0698
	1.4480	2.94	252	.0696
100 ppm	1.7531	3.22	303	.0691
	2.0730	3.90	330	.0637
1000 ppm	1.9255	3.60	309	.0641

Table II.52

Estimation of sitosterol + stigmasterol extracted from hydrolysed tissue grown in the presence of 4-hydroxyisoleucine (Second experiment)

4-Hydroxyisoleucine in the medium	Ratios relative to cholestane				Stigmasterol/ sitosterol	Stigmasterol % w/w m.f.b.	Major sterols % w/w m.f.b. ⁂
	Sit.*	Stig.*	Sit.**	Stig.**			
Control	3.82	1.12	4.90	1.62	.33	.0206	.0831
0 ppm	3.22	1.07	4.87	1.56	.32	.0209	.0865
	4.16	1.19	6.40	1.73	.27	.0194	.0911
10 ppm	3.63	1.02	5.50	1.48	.27	.0188	.0887
	2.94	0.80	4.45	1.16	.26	.0181	.0877
100 ppm	3.22	0.91	4.48	1.32	.27	.0187	.0878
	3.90	1.10	5.91	1.59	.27	.0172	.0809
1000 ppm	3.60	1.01	5.45	1.47	.27	.0173	.0815

* Uncorrected for relative weight response

** Corrected for relative weight response

⁂ Sitosterol + stigmasterol

A COMPARISON OF THE EFFECT OF TWO VITAMIN FORMULATIONS ON THE GROWTH, SAPOGENIN AND STEROL YIELDS OF CULTURES

The B group vitamins have been found in all organisms and are constituents of fundamental tissue enzyme systems involved in the oxidation of foodstuffs.[106] Intact plants are able to synthesise these compounds, but the addition of some B vitamins has been shown to be essential for the maintenance of growth in some plant tissue cultures. Three vitamins are added to most media, namely thiamine hydrochloride, nicotinic acid and pyridoxine hydrochloride.

Thiamine hydrochloride (B_1) was first isolated in 1911 from rice polishings and in animal metabolism has been shown to be a co-enzyme for reactions which involve decarboxylation of acids[106]. Pyridoxine hydrochloride (B_6), its derivatives and their phosphates, act as co-enzymes for many of the metabolic reactions of amino acids, including transamination reactions. Nicotinic acid and nicotinamide were found as constituents of the nucleotides nicotinamide-adenine-dinucleotide (NAD) and NAD phosphate (NADP)[106]. NAD and NADP act as intermediate carriers of hydrogen, released from substrate by dehydrogenase enzymes[106].

Murashige and Skoog[7] added only these three named vitamins to their tobacco callus medium, but other workers have added more complex vitamin formulations in subsequent modifications of the medium. Lin and Staba[107] used the following vitamins with MS medium for the growth of *Mentha piperita* and *Mentha spicata* callus cultures.

Lin and Staba vitamin formulation

Biotin	1.0 mg/litre of media
Choline	1.0
Folic acid	1.0
Nicotinamide	1.0
Pantothenic acid	1.0
Pyridoxal	1.0
Thiamine	1.0
Riboflavin	0.1

Kaul and Staba[50], in work with Dioscorea deltoidea callus and suspension cultures, added a similar vitamin solution to the medium, but included cyanocobalamin. Torrey and Reinert[9] found that omission of riboflavin, calcium pantothenate or biotin from their suspension culture medium impaired the vigorous growth of Daucus carota or Convolvulus arvensis.

Riboflavin has been shown to be a constituent of two co-enzymes involved in carbohydrate metabolism. Flavine mono-nucleotide (FMN) oxides NADH and NADPH. Flavine adenine di-nucleotide (FAD) catalyses the oxidation of aldehydes, D-amino acids and lactic acid.[106] Pantothenic acid has been shown to be a constituent of co-enzyme A and cyanocobalamin(B_{12}) has a role in methylation and transmethylation reactions.[106]

An experiment was performed to study the effect of Kaul and Staba's[50] vitamin formulation on the growth and steroid biosynthesis of Fenugreek tissue cultures. The tissue cultures of Dioscorea deltoidea grown on MS medium in the presence of this formulation had yielded 1% w/w m.f.b. of diosgenin. Tulecke et al[17] had reported the presence of trace amounts of folic acid, riboflavin, biotin and

pantothenic acid in coconut water, but they concluded that at a concentration of 10% v/v in medium, these compounds were at concentrations which would not affect tissue growth. It was thought that the deffiency of an essential vitamin in the medium adopted for Fenugreek tissue cultures might be limiting the biosynthesis of sapogenin.

In the presence of thiamine hydrochloride, pyridoxine hydrochloride, nicotinic acid and the trace amounts of vitamins present in the coconut water added to the medium, the tissue cultures had yielded small amounts of sapogenin. Tissue was grown on two batches of medium containing no coconut water but containing either the simple vitamin formulation of the MS medium or the complex formulation used by Kaul and Staba[50] Table II.53 Both batches of media contained 10 ppm kinetin and 10 ppm NAA as growth regulators and the cultures were grown under the same conditions, at 25° ± 1° with 18 hour photoperiods of warm white fluorescent light. The experiment was continued for three passages of 56 days. The initial inocula for both media were selected from the same stock culture which had been maintained on MS medium with 10% v/v coconut water and 10 ppm NAA.

During the third 56 passage, weighed inocula were grown on the two media and the increase in fresh weight of the cultures compared.

The diosgenin/yamogenin content of the two cultures showed no significant differences over the three passages, Table II.54 The sterol fractions recovered from the hydrolysed tissues were examined and the major sterols estimated, Tables II.55 & 56 The sitosterol component in both cultures was the same in all three

Table II.53

Murashige and Skoog's medium	Vitamins, mg/litre	Kaul and Staba's medium	Vitamins, mg/litre
		Cyanocobalamin	0.0015
		Folic acid	0.5
		Riboflavin	0.5
		Biotin	1.00
		Choline chloride	1.00
		Ca-pantothenate	1.00
Pyridoxine HCl	0.50	Pyridoxine PO_4	1.00
Thiamine HCl	0.10	Thiamine HCl	1.00
Nicotinic Acid	0.50	Nicotinamide	2.00

passages, but the stigmasterol component of the culture grown with the Kaul and Staba[50] vitamins did show a slight increase in the second passage. The mean increase in fresh weight, expressed as a growth index, of cultures grown on the two media were the same, TableII.5? It was concluded that the vitamins included by Kaul and Staba[50] in their medium for Dioscorea deltoidea were unnecessary for the growth of Fenugreek cultures. The biosynthesis of sterols and sapogenins was also unaffected by their inclusion. Fenugreek tissue cultures would appear to be capable of synthesising the co-factors they require and in subsequent work, only the three vitamins of Murashige and Skoog's[7] original formulation were included in media.

Table II.54

Diosgenin/yamogenin yield from tissue grown on MS medium with different vitamin formulations.

Vitamins added	Sample wt.in g	Mean ratio relative to cholestane	Wt. in µg of D + Y	Diosgenin/ yamogenin % w/w of m.f.b.	Mean % w/w of m.f.b.
KS	2.0910 2.142	4.99 4.69	412.0 390.0	.0197 .0180	.0190
MS	2.3846 2.2857	5.86 4.93	480.0 409.0	.0201 .0179	.0190
KS	2.7785 2.4833	5.66 5.55	465.0 455.0	.0167 .0183	.0175
MS	2.1457 2.2830	5.18 5.26	425.0 435.0	.0198 .0190	.0194
KS	2.4610 2.8875	5.60 6.94	460.0 564.0	.0187 .0195	.0191
MS	2.4322 2.0553	5.28 5.28	434.0 434.0	.0178 .0211	.0195

Table II.55

Estimation of sitosterol extracted from hydrolysed tissue

Vitamins added	Sample wt. in g	Mean ratio relative to cholestane	Wt. assayed in µg	Total wt. in µg	% w/w of m.f.b.
1st trial KS	2.0910	7.63	650	2600	0.1244
	2.1642	8.60	732	2928	0.1353
1st trial MS	2.2850	8.14	695	2780	0.1216
	2.3857	9.06	775	3100	0.1300
2nd trial KS	2.7785	9.57	818	3260	0.1173
	2.4833	7.60	645	2580	0.1039
2nd trial MS	2.1457	7.75	643	2572	0.1199
	2.2830	7.39	630	2520	0.1104
3rd trial KS	2.4610	7.40	668	2674	0.1086
	2.8875	9.85	840	3357	0.1163
3rd trial MS	2.4322	8.20	693	2773	0.1140
	2.0553	7.12	612	2450	0.1192

Table II.56

Estimation of stigmasterol + sitosterol

Vitamins added		Mean ratios relative to cholestane				Stigmasterol/ sitosterol	Stigmasterol % w/w m.f.b.	% w/w of m.f.b. ⁂
		Sit.*	Stig.*	Sit.**	Stig.**			
1st trial	KS	7.63	3.63	11.56	5.26	0.46	.0505	0.1750
		8.61	3.50	13.04	5.30	0.40	.0554	0.1908
	MS	8.14	3.34	12.30	4.84	0.39	.0478	0.1695
		9.06	3.90	13.73	5.65	0.41	.0598	0.1898
2nd trial	KS	9.57	5.68	13.86	8.61	0.62	.0727	0.1900
		7.60	3.05	11.01	4.62	0.41	.0422	0.1429
	MS	7.75	2.37	11.23	3.59	0.31	.0383	0.1582
		7.39	2.44	10.71	3.69	0.34	.0375	0.1479
3rd trial	KS	7.40	2.83	10.72	4.28	0.40	.0434	0.1521
		9.85	3.58	14.27	5.42	0.38	.0441	0.1604
	MS	8.20	3.45	11.88	5.22	0.44	.0502	0.1642
		7.12	2.85	10.32	4.33	0.42	.0501	0.1693

* Uncorrected for relative weight response
** Corrected for relative weight response
⁂ Stigmasterol + sitosterol

Table II.57

Growth response of tissues grown on MS medium with different vitamins added

	Murashige and Skoog's vitamins	Kaul and Staba's vitamins
	20.5	33.2
	25.1	31.0
	21.8	18.9
	34.2	12.9
	18.1	20.5
	15.4	22.1
	31.7	31.4
	27.3	19.2
	21.6	20.1
	22.2	24.5
Mean	23.8	23.4

PART II CHAPTER VIII

THE INDUCTION AND MAINTENANCE OF SUSPENSION CULTURES

Street [108] defines a suspension culture as 'cells or cell aggregates dispersed and growing in moving liquid medium'. During incubation the amount of cell material increases for a limited time until the culture reaches a point of maximum yield of cell material. Dilution to the initial cellular content by subculture, and incubation under the same conditions, will result in a similar growth pattern and cellular yield.

Suspension cultures are normally initiated by placing pieces of friable tissue (callus) culture in liquid medium. The subculture is carried out with a pipette or syringe, fine enough to exclude pieces of the initial inoculum. [108]

Suspension cultures overcome many of the inherent disadvantages of solid culture media. Growth response variation, caused by the nutrient gradients between tissue and medium, is eliminated. [108] A similar gradient with respect to gaseous exchange at the occluded base of a tissue culture is also eliminated. Static systems are subject to polarisation by gravity, and the use of pulse or sequential feeding is impossible with solid media. [108] Systems which have been designed for large scale batch or continuous culture are liquid suspension systems and any future commercial scale incubations will be performed with liquid systems. It was, therefore, desirable to determine the conditions for the growth of Fenugreek tissue cultures in suspension culture.

Methods of culture

Several different systems have been developed to achieve movement of the liquid medium, to obtain an even distribution of cells and cell aggregrates, and to promote adequate gaseous exchange between the culture medium and air in the culture vessel. These can be divided into two categories; systems which provide either continuous immersion, or systems which provide periodic immersion of the tissue. In the former, where the tissue is always in contact with the medium, a large gas/liquid interface is required and the medium is shaken, or stirred. Such cultures are grown in flasks which contain liquid medium occupying 20% of their total volume. An orbital shaker operating at speeds between 50 and 100 RPM is often used to suspend the tissue by agitation.

The first system devised for periodic immersion was the Auxophyton, (Stewart Caplin and Millar 1952)[109] fitted with 12.5 cm long, 3.5 cm diameter, tumble tubes containing 10 ml of medium and rotated at 1 or 2 RPM. At 1 RPM the explant spends 2/3rds of the time exposed to the air and the medium flows along the tube during each revolution, maintaining an equilibrium of gaseous exchange. Steward and Shantz[108] later used nipple flasks to culture larger numbers of explants. The flasks have eight or ten nipples and are designed so that the explants distribute themselves in the nipples during rotation and are periodically bathed in medium.

Large batch culture systems have been devised in which gaseous exchange and agitation are maintained by forced aeration, or aeration combined with magnetic stirring.

The stationary culture vessel makes instrumentation and consequent control of the culture conditions possible. More recent work has been carried out on continuous culture systems, which allow the establishment of steady states of growth and metabolism, and these have great potential in the industrial production of secondary plant products. In the two types of system devised, an open continuous system is used, with regulated input of new medium balanced by the harvest of an equal volume of culture.

In the chemostat control system fresh medium input is maintained at a fixed rate; whilst the turbidostat control system maintains the culture at a fixed optical density by intermittent medium input. Wilson et al[110] modified a batch culture system for continucus culture with turbidostat or chemostat control. They found that stable cell densities could be achieved in a chemostat system as a result of an equilibrium established between the dilution rate and the mean cell generation time. The dilution rate determined the limiting nutrient in the culture and it was found that raising the dilution rate resulted in a faster growth rate and a new fixed and lower cell density. A critical dilution rate was achieved when the cell growth rate was at a maximum for the conditions of culture and nutrient ceased to be the limiting factor. At this critical dilution rate a further small increase in dilution rate led to the culture being completely washed from the flask. Using Sycamore cultures Wilson found that at each steady state, achieved by increasing the dilution rate, the light absorption varied within narrow limits and decreased to a uniform value. A turbidostat system, whereby

an electronic control circuit maintained a fixed culture optical density, provided a more critical control mechanism and so overcame the problem of washing out the culture. For a satisfactory flow control employing the turbidostat mechanism, a uniform cell suspension is necessary. Large lumps and aggregates of cells also cause blockage of tubes in culture vessels and present problems of sampling. For a batch culture, or a turbidostat continuous culture process, a fine uniform suspension is, therefore, desirable.

A series of experiments were carried out to try and determine the most suitable conditions for initiating and maintaining a suspension culture of Trigonella foenumgraecum cells. The simplest method of culturing suspensions involves the use of Erlenmeyer flasks shaken on an orbital shaker. Street[108] has described several method by which such flasks may be modified, to produce closed system cultures in which some control of the gaseous phase can be achieved. In all the experiments described in this thesis the gaseous phase was allowed to reach an equilibrium with the atmosphere. Closure of the flasks was by way of non-absorbent cotton wool plugs with aluminium foil caps.

THE SELECTION OF A MEDIUM FOR SUSPENSION CULTURE

Street[108] has stated that although media used in many published systems are capable of supporting growth of the tissue, they do not provide the optimal, or simplest conditions. Modification, or a complete change of medium, is often necessary when cells from a tissue culture clone are transferred to suspension culture conditions. Torry and Reinert[9]

found that the vitamin requirements for rapidly growing Daucus corota and Convolvulus arvensis suspension cultures were different from those of the original tissue culture clone. Street[108] has found that study of the auxin/cytokinin balance of tissue cultures and selection of a combination which provides friable callus can prove useful in designing a suitable medium for suspension cultures. A suitable balance of growth hormones will give an initial inoculum which disperses easily in the agitated liquid medium and often gives a well dispersed suspension.

A series of experiments were carried out using varying concentrations of auxin and cytokinin in Murashige and Skoog's medium to try and initiate a well dispersed suspension system.

(1) The trial of MS medium with 10 ppm NAA and 10% v/v coconut water for the induction of suspension cultures

The solid medium adopted for stock tissue cultures, containing 10 ppm NAA and 10% coconut water, produced a friable callus. An attempt was made to initiate suspension cultures in a liquid form of this medium. Pieces of 30 day old tissue, grown on the solid medium, were placed in 100 ml of liquid medium in each of ten Erlenmeyer flasks (250 ml). The cultures were incubated at 25°, in continuous light, in an orbital incubator. All cultures died within 30 days and it was concluded that either the medium was not suitable for suspension culture, or the tissue used in the incubation was too old.

(2) The trial of a series of concentrations of NAA in MS medium

The concentrations of NAA in the trial media were reduced to 0.01, 0.10, 1.00 ppm and the concentration used in the previous experiment, (10 ppm), repeated. Tissue explants were taken from vigorously growing twenty day old cultures, weighed and transferred aseptically to the media. Ten replicates were grown for each trial for a period of 35 days, under the same conditions as before: cytokinin was provided in a 10% v/v coconut water supplement in each medium.

At the end of the growth period the cultures were harvested, washed and dried. The dry weight of the initial explant material was determined at the beginning of the experiment and growth was expressed as

$$\frac{\text{Final Dry wt. - Initial Dry wt.}}{\text{Initial dry wt.}}$$

In this experiment all the concentrations of NAA supported growth. It was concluded that the use of younger explants enabled growth to be maintained at even the highest auxin concentration of 10 ppm. The type of tissue produced changed with increasing concentration of NAA. Concentrations of 0.01 ppm and 0.1 ppm gave the most dispersed tissue, which ranged from cell suspension, to friable lumps, up to 0.5 cm in cross-section. A concentration of 1 ppm produced less suspension and larger, less friable, pieces. No suspension was apparent in tissue grown on 10 ppm NAA. Hard callus-like pieces were formed up to 2 cm in section and in some flasks the original explant did not disperse at all. No root formation was observed in any of the cultures grown in this experiment.

Table II.53

The growth response to a series of concentrations of NAA in MS medium

0.01 ppm NAA	0.10 ppm NAA	1.00 ppm NAA	10.00 ppm NAA
57.6	112.5	121.0	49.9
151.4	96.7	95.7	48.8
103.3	131.2	93.5	61.8
90.5	88.4	114.7	64.1
144.2	84.1	135.8	58.2
54.2	130.3	106.4	48.9
142.8	118.35	157.9	61.1
121.5	130.2	119.2	81.5
84.1	96.28	105.4	20.0
106.3	128.5	126.3	30.1
$\bar{x}$ 105.6	111.6	117.6	52.4

For the experiment F = 16.04

F = 4.5 at P = .01

FigII.26 Growth response of suspension cultures to different concentrations of NAA and 10% v/v coconut water

The increases in dry weight for the media are shown in Table II.58. The cultures grown on 10 ppm showed significantly smaller increases than the other three media, Fig.II.26.The experiment was terminated after 30 days when tissues grown on 10 ppm NAA showed signs of dying or were dead, although tissues grown on the other three media were still green and healthy.

(3) The trial of a series of coconut water concentration with MS medium

The concentration of NAA (10 ppm) used in stock static cultures was unsatisfactory for initiating suspension cultures. Concentrations of 0.01, 0.1 and 1 ppm had produced some suspension in the presence of a fixed (10% v/v) concentration of coconut water. An experiment was carried out in which the coconut water concentration was varied in the presence of a fixed concentration of auxin, to try and obtain a better dispersion of the cells. Media were prepared containing 0, 5, 10 and 20% v/v coconut water and 1 ppm NAA and cultures were grown, as before, for 32 days.

The results show, TableII.59, that the maximum increase in dry cell weight was obtained with 5 and 10% v/v coconut water and the yields at 0 and 20% v/v were significantly lower Fig.II.27.Absence of cytokinin in the medium resulted in death of the tissues after 20 days. Organogenesis took place in the media containing 5% and 10% v/v and primary roots were formed in seven of the cultures grown on 5% v/v coconut water and three of the cultures grown on 10% v/v coconut water. No root formation was observed in cultures grown on 20% v/v coconut water. The formation of roots indicated that the auxin:cytokinin ratio was high, but increase in the

Table II.59

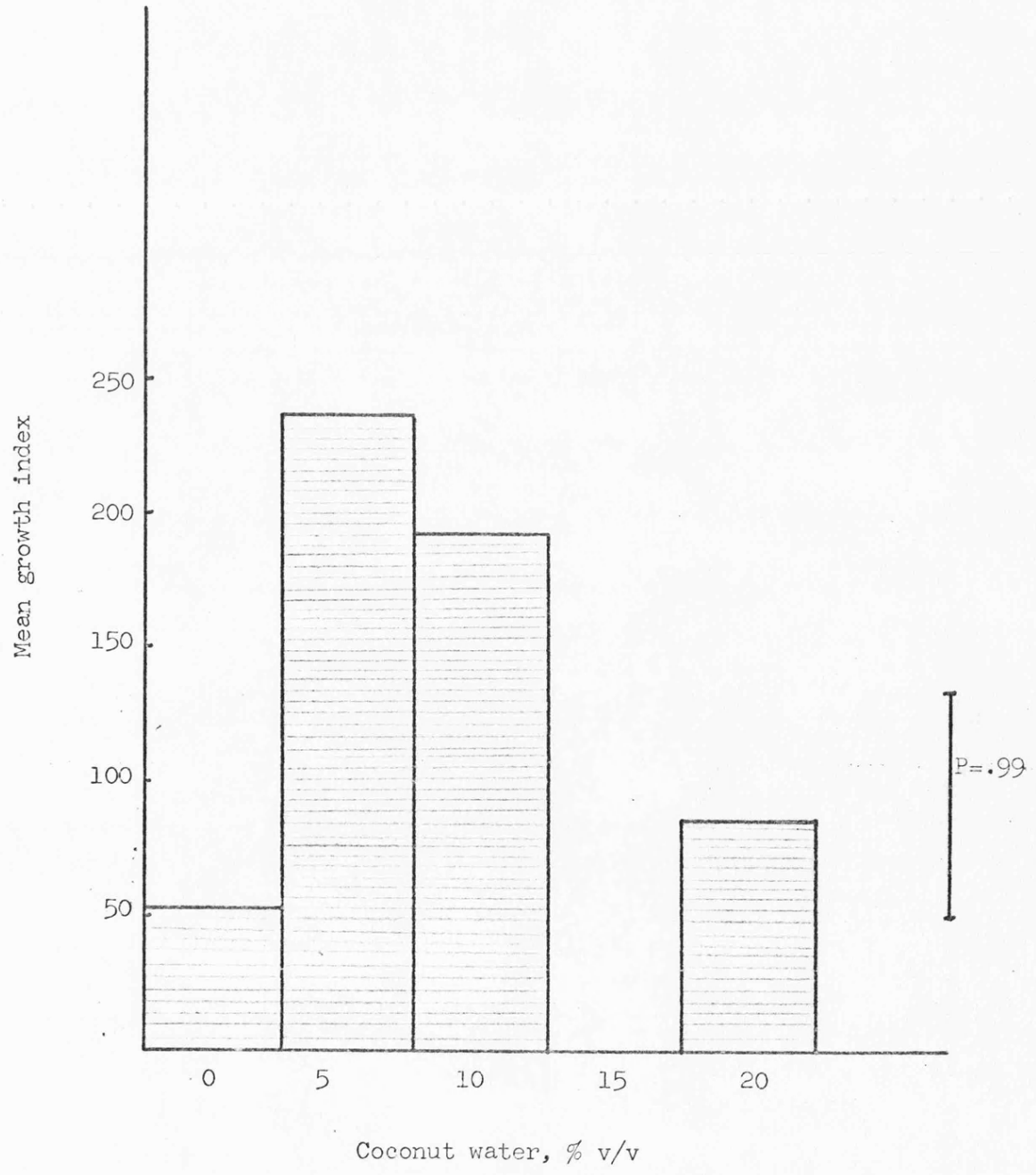
The growth response to a series of coconut water concentrations

Coconut Water 0% v/v	5% v/v	10% v/v	20% v/v
51.9	235.8	113.8	86.3
46.3	287.8	145.1	158.4
28.0	247.7	176.7	54.7
84.0	171.9	212.3	85.9
38.9	318.4	385.5	89.9
62.2	154.1	126.4	150.4
55.9	153.4	194.3	60.1
50.2	255.8	198.3	36.8
58.8	211.9	175.4	64.7
51.5	316.8	200.4	68.1
$\bar{x}$ 52.8	235.4	192.8	85.53

For the experiment F = 26.75

F = 4.5 at P = .01

Fig.II.27 Growth response of suspension cultures to different concentrations of coconut water and 1.0 ppm NAA



cytokinin level, above 10% coconut water, resulted in poor growth. It was concluded that a concentration of 1 ppm NAA was too high for suspension culture and that concentrations of greater than 10% v/v coconut water were inhibitory to growth.

No reason could be found for the lack of root formation in the previous experiment on media containing 1 and 10 ppm NAA in the presence of 10% v/v coconut water.

(4) The trial of a series of kinetin concentrations

The concentration of NAA was reduced to 0.1 ppm in this experiment. The presence of 20% v/v coconut water had proved inhibitory to growth, but because of the undefined nature of the supplement, it was not known if inhibition was caused by excess cytokinin, or the presence of other compounds. Coconut water was, therefore, replaced by a range of concentrations of kinetin (6-furfurylaminopurine). The type of tissue and growth on 0.01, 0.1 and 1 ppm kinetin were compared with a control grown on 10% v/v coconut water after 35 days growth.

No organogenesis was observed in tissues grown on the reduced concentration of NAA (0.1 ppm). The dry cell weight increase obtained, TableII.60, with the medium containing 1 ppm of kinetin was significantly greater than that obtained with the other media. The growth of tissues in medium containing 0.1 ppm kinetin was similar to that in medium containing 10% v/v coconut water. A concentration of 0.01 ppm kinetin gave significantly less increase in dry cell weight, Fig.II.28. The type of tissue produced by all the media was similar, consisting of a mixture of aggregate sizes from suspension, to lumps 0.5 cm in cross-section. Chlorophyll was produced by the cells of all the cultures.

Table II.60

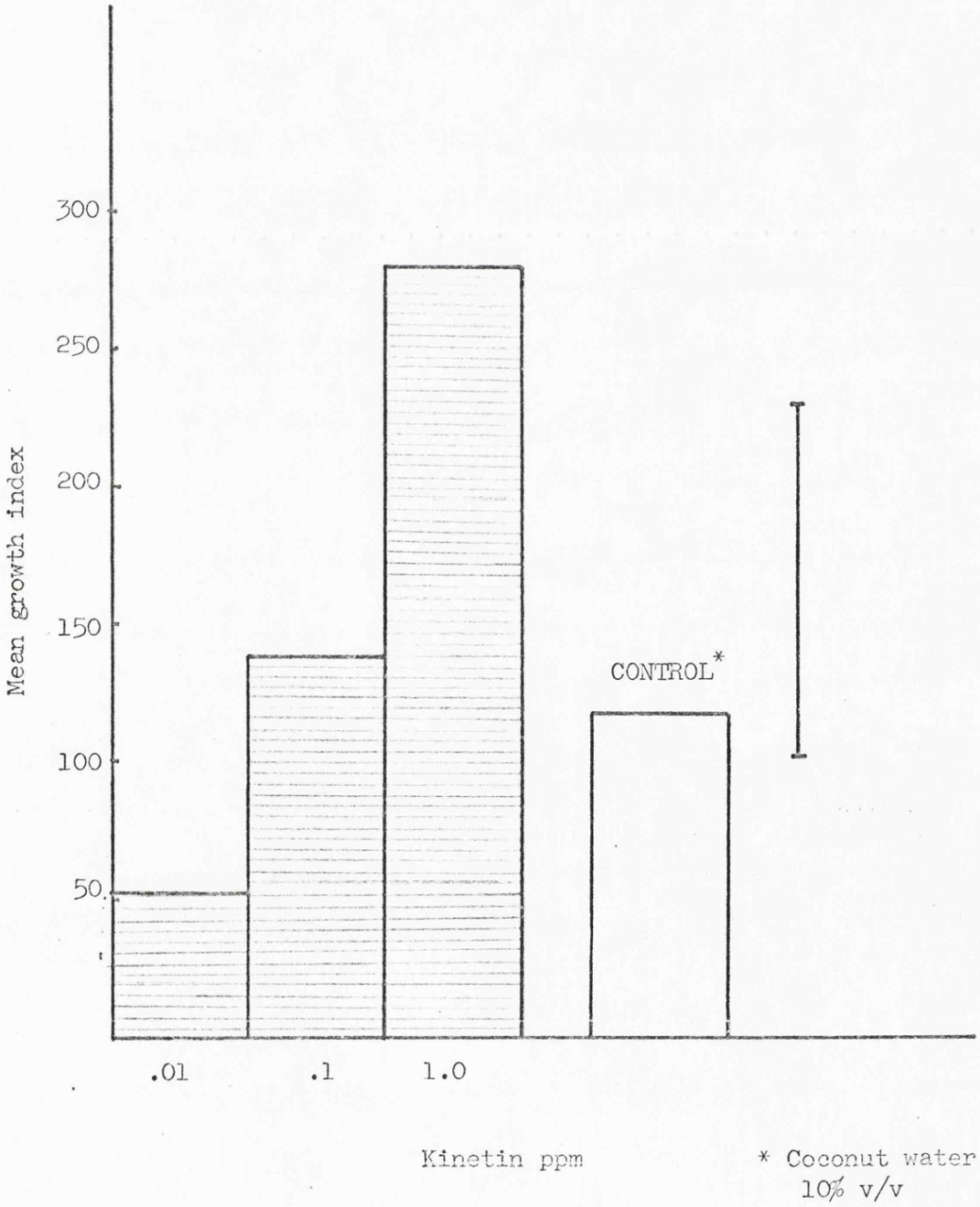
The growth response to a series of kinetin concentrations in the induction of suspension cultures

.01 ppm kinetin	0.1 ppm kinetin	1.0 ppm kinetin	10% v/v coconut water
40.3	46.5	549.1	236.6
62.1	163.6	169.5	52.4
49.6	73.6	296.6	185.7
58.5	101.6	218.3	137.8
73.1	143.5	222.2	58.5
44.4	148.8	229.2	85.9
90.8	69.6	113.4	102.6
41.9	357.4	409.8	46.9
15.2	122.1	298.7	152.1
54.5	124.8	271.8	118.4
$\bar{x}$ 53.00	135.14	282.36	117.69

For the experiment F = 12.4

F = 4.5 at P = .01

Fig II. 28 Growth response of suspension cultures to different concentrations of kinetin and 0.1 ppm NAA



(5) The trial of larger kinetin concentrations in MS medium

Trial media were prepared containing 1, 2, 4 and 8 ppm kinetin and 0.1 ppm NAA. These were inoculated with weighed tissue culture fragments grown as before for a period of 35 days. The increases in dry cell weight were compared with tissue grown on 10% v/v coconut water and 0.1 ppm NAA.

There was no significant difference in the dry cell weight increase at the different concentrations of kinetin (see Table II.6I). Increase in the kinetin concentration did not produce a consequent increase in growth (Fig.II.29). All the trial cultures showed a greater growth than cultures grown with 0.1 ppm NAA and 10% v/v coconut water.

The cultures grown on 1 and 2 ppm of kinetin produced the most dispersed tissue.

Summary

From the results of these experiments it was concluded that an NAA concentration of less than 1 ppm was required for the initiation of undifferentiated suspension cultures. Concentrations of 1 ppm or greater resulted in cultures which produced large lumps and had a tendency to differentiate to form roots.

It was found that a supplement of the coconut water was inhibitory to growth at concentrations greater than 10% v/v and high dry weight increases were obtained when the coconut water was replaced by kinetin. Increase in the kinetin concentration beyond 1 ppm caused no increase in growth rate but at concentrations up to 8 ppm tissues grew healthily with no signs of differentiation and shoot formation.

Table II.6I

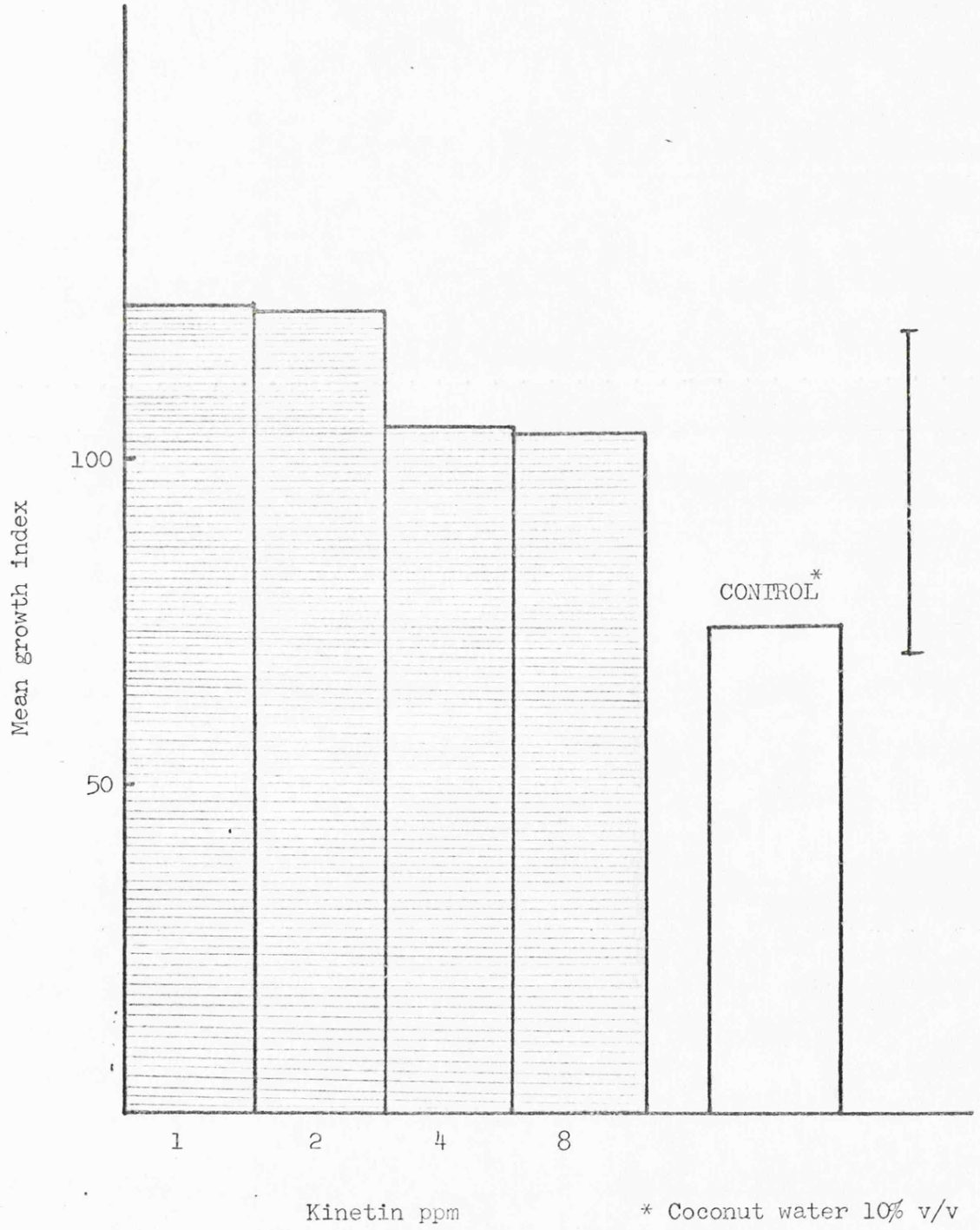
The growth response to a series of kinetin concentrations in the induction of suspension cultures

1 ppm kinetin	2 ppm kinetin	4 ppm kinetin	8 ppm kinetin	10% v/v coconut water
81.9	99.0	83.2	128.3	31.7
154.1	108.6	101.4	97.8	33.5
243.5	149.6	91.2	62.4	98.6
108.3	158.8	149.0	122.4	102.0
82.6	158.8	130.8	83.6	99.6
210.5	94.9	85.0	94.0	34.4
73.0	146.2	87.5	112.2	81.3
154.2	133.7	96.8	102.9	89.4
78.9	113.9	156.0	75.2	95.5
115.6	102.5	97.6	143.8	100.5
89.6	108.0	80.4	115.2	88.1
101.1	102.9	103.8	109.9	35.0
$\bar{x}$ 124.4	123.1	105.2	104.0	74.0

For the experiment F = 4.2

F = 3.7 at P = 0.01

Fig.II.29 Growth response of suspension cultures to different concentrations of kinetin and 0.1 ppm NAA



None of the trial media resulted in the formation of a uniform suspension in the first passage but 0.1 ppm NAA and 1 or 2 ppm kinetin gave cultures in which the tissue was most dispersed.

(6) Alteration of the vitamin content of the suspension culture medium

Torrey and Reinert[9] found that the addition of certain vitamins was essential when suspension cultures were cultivated from callus cultures of Daucus carota and Convolvulus arvensis. on media containing no complex natural supplements. The omission of calcium pantothenate, biotin or riboflavin from the medium caused a marked decrease in growth. Addition of the complex vitamin formulation, adopted by Kaul and Staba[50] for Dioscorea cultures, had given no significant difference in the growth of Fenugreek callus cultures grown on a medium containing coconut water (Table II.3). The medium adopted for suspension cultures contained no coconut water and the Kaul and Staba vitamin supplement was added to see if change in the growth response occurred.

A batch of MS medium containing 0.1 ppm NAA and 2 ppm kinetin was prepared and to half was added the Kaul and Staba[50] vitamin solution (see page I99) and to the other half, the vitamins of the normal MS formulation. Known weights of callus were added to ten flasks of each medium and incubated at 25° in an orbital incubator. The cultures in the medium containing the normal MS vitamin formulation grew, but the

tissues in the trial medium all died with little or no growth.

The only differences between the two media were in the vitamins added and it was concluded that the increased availability in liquid medium of substances, caused one or more of the components of the Kaul and Staba formulation present at a toxic level. No further work was carried out with different vitamin formulations in liquid media and the original MS medium vitamins were used for subsequent work.

THE MAINTENANCE OF AN ESTABLISHED SUSPENSION CULTURE

From the results obtained in the preliminary experiments a medium containing 0.1 ppm NAA and 2 ppm kinetin was chosen for the establishment of continuous suspension cultures of Fenugreek. The stock callus culture, grown on MS medium with 10 ppm NAA and 10% v/v coconut water, had consistently yielded 0.02-0.03% m.f.b. diosgenin/yamogenin and it was used to initiate the suspension cultures. About 300 mg of fresh callus was added to each of 10 flasks of medium and grown in continuous white fluorescent light at 25° in an orbital incubator. As in previous experiments, the tissue grew well on this medium and produced cell aggregates and cell suspension. Subculturing was performed after 30 days and samples of both suspension and small aggregates, were transferred to flasks of fresh medium in about 4 ml of the exhausted medium. Only a small amount of tissue was used for each inoculation, in an attempt to obtain a well dispersed tissue growth and although all the subcultures grew, a period of 60 days was needed before a satisfactory yield of tissue was achieved. Tissue growth again consisted of a suspension and aggregates up to

Table II.62

The diosgenin/yamogenin yield from Fenugreek suspension cultures

	Sample wt. in g	Mean ratio relative to cholestane	Weight diosgenin/ yamogenin µg	Diosgenin/ yamogenin % w/w m.f.b.
First 30 day growth period	2.3438	1.78	160	.0067
	2.3251	1.30	124	.0053
Second 60 day growth period	2.5619	1.50	138	.0054
	2.4647	1.38	128	.0052

0.5 cm in cross-section. A second subculture was successfully performed and the cultures were growing at the time of writing (July 1974). The nature of the cultures has changed and smaller, more friable aggregates and more cell suspension has been formed.

The diosgenin/yamogenin yield of suspension culture from the first and second growth periods was assayed. The recovery of sapogenin from the tissue was very low and the amount detected is estimated in Table II.62. It was concluded that although the medium used was satisfactory for the growth of suspension cultures it was unsatisfactory for the accumulation of sapogenin. Callus culture tissue producing 0.02-0.03% w/w m.f.b. diosgenin/yamogenin produced even less (0.005-0.006% m.f.b.) when grown in the liquid medium. Further investigation of the conditions which would encourage both vigorous growth and the accumulation of sapogenins in suspension cultures was not possible in the time available.

REFERENCES

PART 2 - RESULTS AND DISCUSSION

1. Sunderland, N. and Wells, B. Ann. Bot. 1968, 32, 327-346.

2. Carew, D.P. and Staba, E.J. Lloydia, 1965, 28 (1), 1-26.

3. Pollard, J.K., Shantz, E.M. and Steward, F.C. Plant Physiol., 1961, 36, 482-501.

4. Risser, P.G. and White, P.R. Physiol. Plantarum, 1964, 17, 620-635.

5. Street, H.E. Plant tissue and cell culture, 1973, pp 1-30, Ed. H. E. Street, Blackwell Press.

6. Dutta, T.R. and Mc. Ilrath W.J. Botan. Gaz., 1964, 125, 89-96.

7. Murashige T. and Skoog, F. Physiol. Plant., 1962, 15, 473-497.

8. Lin M. and Staba, E.J. Lloydia, 1961, 24, 139-145.

9. Torrey, J.G. and Reinert, J. Plant Physiol., 1961, 36, 483-491.

10. Tulecke, W. Plant Physiol., 1960, 35, 19-24.

11. Straus, J. Am. J. Botany, 1960, 47, 641-647.

12. Steward, F.C. and Shantz, E.M. Ann. Rev. Plant Physiol., 1959, 10, 379-401.

13. Skoog, F. and Miller, C. O. Symp. Soc. exp. Biol., 1957, 11, 118-130.

14. White, P.R. Plant Physiol. Lancaster, 1934, 9, 585-600.

15. Harris, G.P. Nature, 1953, 172, 1003-1007.

16. Harris, G.P. New Phytologist, 1956, 55, 253-268.

17. Tulecke, W., Weinstein, L., Rutner, A. and Laurencot, H. 1961, Boyce Thompson Inst. Contributions, Vol.21, 115-128.

18. Kefford, N.P. and Goldacre, P.L. Am. J. Bot., 1961, 48, 643-650.

19. Hildebrandt, A.C., Riker, A.J. and Duggar, B.M. Amer. J. Bot., 1946, 33, 591-597.

20. Sunderland N, John Innes Inst., Colney Lane, Norwich. Private correspondence, 1970.

21. Toplin I. and Gaden, E.L. J. Biochem. Microbiol. Technology & Engineering, 1961, 3, 311-323.

22. Toplin, I. Biotechn. Bioeng., 1962, 4, 331-340.

23. Street, H.E., Raj Bhandary, S.B. Collin, H. A. and Thomas, E. Ann. Bot., 1969, 33, 647.

24. Krikorian, A.D. and Steward, F.C. Plant Physiology, Vol. VB, pp 227-326, Ed. F. C. Steward, Academic Press, New York, 1969.

25. Carceller, M., Davey, M.R., Lowler, M.W. and Street, H.E. Protoplasma, 1971, 73, 367-385.

26. Constabel, F., Skyluck, J. and Gamborg, O. Planta, 1971 96, 306-316.

27. Kaul, B., Stohs, S.J. and Staba, E.J. Lloydia, 1969, 32 (3), 347-359.

28. Turner, T. The Pharmaceutical Journal, 1971, 341-344 (May).

29. Caplin, S. and Steward, F. Ann. Bot., 1952, 16, 491-504.

30. Morel G. and Wetmore R. Am J. Bot., 1951, 38, 138-140.

31. Nitsch, J. SCI Monograph, No.31, page 111. 1968,

32. Paris, D., Duhamet, L. and Goris, A. Compt. Rend. Soc. Biol., 1953, 236.

33. Heble, M.R., Narayanaswami, S. and Chahda, M.S. Phytochem., 1971, 10, 2393-2394.

34. Tomita, Y., Uomori, A. and Minato, H. Phytochem., 9, 111-114. 1970.

35. Mehta, A. and Staba, E.J. J. Pharm. Sci., 1970, 59 (6), 864-65.

36. Khanna, P., Mohan, S., Nag, T.N. Lloydia, 1971, 34 (1), 168-169.

37. Khanna, P. and Jain, S.C. Lloydia, 1973, 36 (1), 96-98

38. Brain, K.R., Lockwood, G.B. and Turner, T.D. Abstract - FMAKAZ, 1973, 82, 5.

39. Yeoman, M.M. Plant tissue and cell culture, 1973, p.41, Ed. H.E. Street, Blackwell Press, Oxford.

40. Reinert, J. Plant tissue and cell culture, 1973, p.339, Ed. H.E. Street, Blackwell Press, Oxford.

41. Skoog, F. and Miller, C. O. Symp. Soc. Exp. Biol., 1957 11, 118-130.

42. Torrey, J.G. Cell, organism and milieu, 1961, pp, 189-222, Ed. D. Rudnick, Ronald Press, New York.

43. Gautheret, R.J. La culture des tissus végétaux, 1945, Gallinard, Paris.

44. Skoog, F. Am. J. Bot., 1944, 31, 19-24.

45. Les cultures de tissus de plantes, pp.187-199 Gautheret R.J. Colloques Internationaux de CNRS, No.193, Paris, 1971.

46. MacMillan, J. Aspects of terpenoid chemistry and biochemistry, 1971. Ed. T. W. Goodwin, Academic Press, New York.

47. Robinson, D.R. and Ryback, G. Biochem. J., 1969, 113, 895.

48. Chen, C.M. and Hall, R.H. Phytochem., 1969, 8, 1687-1695.

49. McChesney, J.B. Can. J. Bot., 1970, 48, 2357-2359.

50. Kaul, B. and Staba, E.J. Lloydia, 1968, 31, 171-179.

51. Geuns, J.M. Phytochem., 1973, 12, 103-106.

52. Jefferies, T.M. Ph.D. Thesis, Bath University, 1973.

53. Grunwald, C. J. Chromatog., 1969, 44, 173-175.

54. Eddy, C.R., Wall, M.E., Klumpt,M. and Scott, M. Anal. Chem., 1953, 25, 266-271.

55. Wall, M.E., Eddy, C.R., McClennan, M.L. and Klumpt, M.E. Anal. Chem., 1952, 24, 1337-1341.

56. Wood, C. Ph.D. Thesis, Bath University, 1970.

57. Marker, R.E., Turner, D.L. and Ulshafer, P.R. J. Am. Chem. Soc., 1940, 62, 2542-3.

58. Rothrock, J.W., Hammes, P.A. and McAleer, W.J. Ind. Eng. Chem., 1957, 49, 186-188.

59. Bedour, M.S., El-Munajjed, D., Fayez, M.B.E. and Girgis, A.N. J. Pharm. Sci., 1964, 53 (10), 1276-1278.

60. Yamauchi, T. Chem. & Pharm. Bull., 1959, 7 (3).

61. Blunden, G., Hardman, R. and Morrison, J.C. J. Pharm. Sci., 1967, 56, 948-950.

62. Fazli, F.R.Y. and Hardman, R. Phytochem., 1971, 10, 2497-2503.

63. Miller, M. L. Ph.D. Thesis, Bath University, in preparation.

64. Sofowora, E. A. Ph.D. Thesis, University of Nottingham, 1967.

65. Veigiyfalvi, D., Maroti, M. and Tetenyi, P. Phytochem., 1971, 10, 1389-1390.

66. Fazli, F.R.Y. Ph.D. Thesis, University of Nottingham, 1967.

67. Brain, K.R., Fazli, F.R.Y., Hardman, R. and Wood, A.R. Phytochem., 1968, 7, 1815-1823.

68. Blunden, G., Hardman, R. and Morrison, J. Pharm. Sci., 1967, 56 (8), 948-950.

69. Brain, K. R. and Hardman, R. J. Chromatog., 1968, 38, 355-363.

70. Wielens, H.A., Turner, A. Jr. and Wall, M.E. Anal Chem., 1954, 26, 325-329.

71. Yamagishi, M. and Nakamura, I. Chem. Pharm. Bull., 1958, 16, 421-425.

72. Sanchez, G.L., Acevedo, J.C.M. and Soto, R.R., Analyst, 1972, 97, 973-976.

73. Slack, S.C. and Mader, W.J. Anal. Chem., 1961, 33 (4), 625-627.

74. Hardman, R. and Jefferies, T. M. Planta Medica, 1972, 22 (1), 78-87.

75. Netting, A.G. J. Chromatog., 1970, 53, 507-516.

76. Tomita, Y. and Uomori, A. Chem. Commun., 1971, 284.

77. Cooke, B.K. Analyst, 1970, 95, 95-97.

78. Rozanski, A. Analyst, 1972, 97, 968

79. Hilton, J. Jansen L. and Hull H. Plant Physiol. 14, 353-384 1963.

80. Heftmann, E., Lipids, 6 (2), 128-133. 1971.

81. Ansell, G.B. and Hawthorne, J.N. Phospholipids, chemistry, metabolism and function, Chap.10. Elsevier, Amsterdam. 1964.

82. Sietsma, J. and Haskins, R. Can. J. Microbiol., 13, 361-5. 1967.

83. Sietsma, J. and Haskins, R. Can. J. Biochem., 46, 813.

84. Evans, F.J. Planta (Berl.), 1973, 111, 33-40.

85. Love, A. and Love, D. Arkiv. Bot., 32A, 1-18.

86. Bonner, J., Heftmann, E. and Zewart, J.A.D. Plant Physiol., 38, 81-88. 1963.

87. Kefeli, V.I. Fiziologiya Rastenii, 1970, 18 (3), 614-630.

88. Rypacek, V. and Sladky, Z., Mycopathologia et Mycologia applicata, 1972, 46 (1), 65-72.

89. Olsen, R.A. Physiol. Plantarum, 1973, 28 (3), 507-515.

90. Elliot, C.G., Hendrie, M. and Knights, B.A. J. gen. Microbiol., 1966, 42, 425-435.

91. Kopcewicz, J. Acta Societatis Botanicorum Poloniae, 1970, 39 (2), 339-346.

92. Bush, P.B. and Grunwald, C. Plant Physiol., 1972, 50, 69-72.

93. Ingrams, D.S., Knights, B.A., McEvoy, I.J. and McKay, P. Phytochem., 1968, 7, 1241-1245.

94. Roddick, J.G. and Butcher, D.N. Phytochem., 1972, 11, 2991-2997.

95. Elliott, C.G. J. gen. Microbiol., 1969, 56, 331-343.

96. Evans, F.J. J. Pharm. Pharmac., 1972, 24, 645-650.

97. Kemp. R.J., Goad, L.J. and Mercer, E.I. ibid, 6, 1609-1615, 1967.

98. Ibrahim, R.K., Thakur, M.L. and Permanand, B. Lloydia, 1971, 34 (2), 175-182.

99. Thomas, D.R. Phytochem., 1970, 9, 1443-1451.

100. Tomita, Y., Uomori, A. and Minato, H. Phytochem., 1970, 9, 111-114.

101. Heble, M.R., Narayanaswami, S. and Chahda, M.S. Die Naturwissenschaften, 1968, 55 (7), 350-351.

102. Sandstedt, R.M. and Skoog, F. Physiol. Plantarum, 1960, 13, 250.

103. Yeoman, M.M. and Aitchison, P.A. Plant tissue and cell culture, Chap.10, pp 240-268. Ed. H. E. Street, Blackwell Press, Oxford, 1973.

104. Fowden, L., Pratt, H. and Smith, A. Phytochem., 1973, 12, 1707-1711.

105. Supniewska, J.H. and Dohnal, B. Dissert. Pharm. Pharmacol., 1972, 24, 2.

106. Samson Wright's Applied Physiology, Edition 11, p.424. Oxford Medical Publications.

107. Lin, M-L. and Staba, E.J. Lloydia, 1961, 24, 139-145.

108. Street, H. E. Plant tissue and cell culture, pp 59-100. Ed. H. E. Street, Blackwell Press, Oxford, 1973.

109. Steward, F.C., Caplin, S.M. and Millar, F.K. Ann. Bot., 1953, 16, 58-77.

110. Wilson, S.B., King, P.J. and Street, H.E. J. exp. Bot., 1971, 21, 177-207.

111. Blunden, G., and Rhodes, C.T., J. Pharm. Sci., 1967, 56, 602-604.

EXPERIMENTAL

PART III CHAPTER I

THE INDUCTION OF FENUGREEK TISSUE CULTURES

MS TOBACCO TISSUE CULTURE MEDIUM

(1) The composition of stock solutions for MS medium

The ingredients listed in the macronutrients, Table III.1, were dissolved in distilled water and the solution made up to 500 ml and stored in reagent bottles in a refrigerator. The solutions were inspected at weekly intervals for biological contaminants and replaced if necessary. After a maximum storage period of two months unused solutions were discarded. A 2 l. stock solution of the minor inorganic nutrients was prepared containing the quantity of each compound required for a 2 l. batch of medium in 10 ml. The solution was dispensed into 10 ml amber glass ampoules, which were sealed and autoclaved at 15 psi for 15 minutes. Similarly, a stock solution of a mixture of glycine and myo-inositol was prepared and stored in 10 ml ampoules. Fresh vitamin stock solution was usually prepared for each batch of medium, but was stored for up to three days at 2-4^{o} on some occasions. Thiamine hydrochloride (10 mg) was dissolved in distilled water and diluted to a volume of 10 ml in a volumetric flask. One ml was transferred to a 100 ml volumetric flask and pyridoxine hydrochloride (5 mg) and nicotinic acid (5 mg) added. The solution was made up to volume and 10 ml added to 1 litre of medium, after membrane filter sterilisation. The vitamin solution was added to cold sterile liquid media or, to media containing agar, when the autoclaved flask was warm to the touch.

Disodium ethylenediamine tetra-acetate solution (Na_2EDTA) was prepared in 500 ml stock quantities (7.46 g/l.). Ferrous sulphate (5.56 g/l.) solution was freshly prepared for each batch of medium. For a 2 l. batch of medium 10 ml of each of these two stock solutions (Table III) were mixed 30 minutes before being added to the medium.

The coconut water, used as a medium supplement, was prepared by the method recommended by Street. [I] A commercial sample of 100 mature coconuts was obtained and the liquid endosperm drained from each nut and examined for biological contamination before being bulked. About 8 l. of coconut water was obtained and this was immediately boiled under reflux for 30 minutes, to denature the proteins present, and cooled. The liquid was filtered, through a filter bed of hyflo supercel using vacuum. The filtrate was mixed thoroughly before being dispensed in 100 ml aliquots into screw-cap 100 ml medical flats and autoclaved at 15 psi for 15 minutes. The sterile coconut water was stored at 2-4°.

Oxoid No.1 agar was used in most of the work although Difco Bacto-Agar was used in some later experiments. A concentration of 1.0 % w/v was satisfactory with Oxoid No.1 and 1.2% w/v with Difco Agar. Analar sucrose was used for all media. A stock solution of NAA was prepared by heating 100 mg of NAA with 300 ml of water, under reflux, until the solid dissolved. The cooled solution was made up to 500 ml. A similar procedure was adopted with 2,4-D. Unused auxin solutions were discarded after one month.

Table III.I

Stock solutions used in the preparation of MS medium

Macronutrients

	g/500 ml of stock solution	ml of stock sol./ l. of medium
NH_4NO_3	87.5	10
KNO_3	95.0	10
$MgSO_4 7H_2O$	18.5	10
KH_2PO_4	8.5	10
$CaCl_2 2H_2O$	22.0	10

Micronutrients in a 2 l. stock solution

	g/2 l. of stock solution	ml of stock sol./ l. of medium
H_3BO_3	2.480	5
$MnSO_4$	8.920	
$ZnSO_4 7H_2O$	3.440	
KI	0.332	
$Na_2MoO_4 2H_2O$	0.100	
$CuSO_4 5H_2O$	0.010	
$CoCl_2$	0.010	

	g/2 l. of stock solution	
Myo-inositol	40	5
Glycin	0.8	

Vitamin stock solution

	mg/100 ml stock solution	ml/l. of medium
Pyridoxine HCl	5	10
Nicotinic acid	5	
Thiamine HCl	1	

Iron Chelate

	g/500 ml stock solution	
Na_2EDTA	3.73	5
$FeSO_4 7H_2O$	2.78	5

The pH of the media was adjusted to 6 by the addition of 1N potassium hydroxide solution before autoclaving. A Pye model 79 pH meter fitted with a Pye Ingold pH electrode (model 401) was used for pH measurement.

EXPERIMENT TO STUDY CHANGE IN pH OF MEDIA AFTER AUTOCLAVING

A 1 litre batch of MS medium containing 10 ppm NAA and 10% v/v coconut water was prepared from the stock solutions, TableIII.I. The medium was mixed thoroughly and 9 aliquots of 100 ml taken. The pH of the medium was 5.25 and each batch was adjusted with either 1N potassium hydroxide solution or 1N hydrochloric acid to give a series of media with pH values of: 5.20, 5.39, 5.64, 5.89, 5.99, 6.24, 6.43, 6.63 and 6.80. The pH determinations were carried out on a Radiometer Copenhagen pH meter, type PHM 26C, fitted with a type G202C glass electrode. The instrument was adjusted for pH measurement with increased accuracy of reading.

Clean 100 ml medical flats were rinsed with distilled water and dried. These were filled with the media (100 ml) and autoclaved in a Gallenkamp portable electric autoclave for 15 minutes at 15 psi. The autoclaved media were cooled to room temperature and the pH re-measured. A graph of pH after autoclaving against pH before autoclaving was plotted.

ASEPTIC MANIPULATIONS

All aseptic manipulations were performed in a Pathfinder laminar flow, clean air cabinet (Type 63T) fitted with a Fram 3 filter unit, complying with U.S.A. federal standard 209A Class 100, with an air flow of 90 ft/minute. The air flow was switched on at least 20 minutes before use and the

cabinet swabbed with absolute alcohol immediately prior to use. Forceps and scalpels were heated with a spirit burner and quenched in sterile distilled water before use. Disposable plastic gloves, swabbed with absolute alcohol, were worn at all times during aseptic work. Sterile transparent (100 ml) plastic jars, fitted with metal screw-cap lids, (Richardson, Leicester) were used extensively as callus culture containers.

SEED SURFACE STERILISATION

A sample of 20 seeds, Ethiopian type, RH.2602, was immersed in 25 ml of bromine water for 2 minutes, in a sealed sterile plastic jar (100 ml). The sample was transferred to a second jar containing 25 ml of 10 volume hydrogen peroxide solution and left for a further 10 minutes. The seeds were then washed with two 25 ml quantities of sterile distilled water, to remove all traces of the reagents.

A 200 ml batch of MS medium, containing no auxin or vitamins, was prepared and warmed with 1% w/v agar until the agar was in solution. Twenty ml of medium were poured into each of 10 glass petri dishes (BS611) which were autoclaved. Two of the treated seeds were aseptically transferred to each petri dish of cold medium and incubated at 25° (L.E.E.C. PC2 incubator). The seeds were examined daily for fungal growth and germination and the experiment terminated after 5 days.

A 100 ml volume of 1% aqueous solution of β-propiolactone (BDH) was prepared and 30 ml (approx.) immediately added to 20 seeds, type RH.2602, in a sterile plastic jar. The container was sealed and placed in an oven at 50° for 30 minutes, with agitation every 10 minutes. The seeds were washed twice

with sterile distilled water, and then incubated on the MS medium at 25° for seven days. The seedlings were examined for fungal contamination and damage by the sterilising agent.

A 20 seed sample of Moroccan seed, type RH.2336, was similarly treated with β-propiolactone and incubated for seven days on MS medium. Treatment with a 1% aqueous solution of β-propiolactone for 30 minutes, at 50°C, was adopted as a standard procedure for seed sterilisation.

THE INDUCTION OF CALLUS CULTURES FROM STERILE COTYLEDONS

A sample of 10 selected seeds, of Moroccan type RH.2336, was surface sterilised. Ten glass petri dishes containing 1% w/v agar in sterile distilled water were prepared and one seed incubated in each dish at 25° in the dark for five days. A 200 ml batch of MS medium (10 ppm NAA 10% v/v coconut water pH6) containing 1% w/v of agar was autoclaved at 15 psi for 15 minutes. Vitamin solution, sterilised by membrane filtration, was added to the cooled medium and the medium aseptically poured into 10 sterile plastic jars (20 ml/jar). The cotyledons were removed from the seedlings and one pair added to each jar of medium. The jars were sealed and five cultures grown for 30 days in continuous light (Ediswan 'warm white' 65/80W fluorescent tubes) and five in total darkness. The light intensity at the shelf levels in the incubator room was adjusted to 1500-1800 Lux using a E.E.L. light meter and the room temperature maintained at 25° ± 1° with a fan heater and water-cooled radiator.

The undifferentiated tissue which formed at the cut edges of the cotyledons was removed after 30 days and approximately

50 mg subcultured into each of a series of jars containing fresh, sterile MS medium containing 10 ppm NAA and 10% v/v coconut water.

PART III CHAPTER II

THE DETERMINATION OF THE OPTIMUM CONDITIONS FOR FENUGREEK TISSUE CULTURE GROWTH

GROWTH RESPONSE OF FENUGREEK TISSUE CULTURE TO DIFFERENT COMBINATIONS OF GROWTH REGULATOR

(1) Growth response on the induction medium

Twenty 100 ml sterile plastic jars were calibrated for a volume of 20 ml, to ensure uniformity of filling with medium. A 20 ml volume of water was added to a jar and the depth of the liquid measured. A similar depth was measured and marked on the twenty jars to be used in the experiment.

A 100 ml vitamin solution, see TableIII.I, and a 500 ml batch of MS medium (10 ppm NAA, 10% v/v coconut water pH 6, hereafter referred to as R.S. medium) were prepared. After adjustment of the pH to 6 with 1N potassium hydroxide solution and addition of 1% w/v of agar,the medium was autoclaved at 15 psi for 15 minutes. An Everett 10 ml,Luerlock syringe, fitted with a Sartorious membrane filtration unit, (0.45μ pore) was used to introduce 5 ml of the vitamin solution (page228) into the autoclaved medium when the flask was cool enough to be handheld. The vitamin solution and medium were mixed by swirling the flask and poured into the plastic jars and allowed to set. Another 20 sealed sterile jars were tared and coded. A healthy 20 day-old subculture of callus, maintained for 5 months on R.S. medium and grown in continuous warm white light at 25°C,was used to provide explants. Ten uniform explants, each approximately 50 mg in weight, were each placed in an empty, tared plastic jar and weighed. Ten jars

of media were inoculated with the tissue and each coded to correspond with the tared jar from which the tissue was taken. The empty tared jars were aseptically re-sealed and retained for subsequent weighings. The procedure was repeated for the other jars of medium with ten pieces of tissue grown in the dark for the same length of time on the R.S. medium. The cultures were grown under the conditions employed for the original stock cultures and were weighed at intervals during the growth period. The growth index was calculated for each tissue, after each weighing, from the formula:

$$\frac{\text{Weight of tissue - Initial Wt.}}{\text{Initial Wt.}}$$

A graph was plotted of the sample mean growth index against time for the two sets of replicates and the 95% confidence interval calculated for the population mean.

(2) Variation of the NAA concentration in the induction medium

The nutrient stock solutions and sucrose (page228) for a 1 litre batch of M.S. medium, including a 10% v/v coconut water supplement, were made up to 500 ml with distilled water, mixed thoroughly and divided into 5 batches each of 100 ml. A stock solution of 200 mg/l NAA (Sol.A) was diluted to give two solutions of 20 mg/l (Sol.B) and 2 mg/l (Sol.C). To two batches of the nutrient concentrate were added 20 ml and 10 ml of solution A. To one batch was added 10 ml of solution B and to the two remaining batches 10 ml and 1 ml of solution C. All the nutrient concentrates were made up to 200 ml with distilled water, the pH of each adjusted to 6 with 1N potassium hydroxide solution, and 1% w/v of agar added. The media were

autoclaved at 15 psi for 15 minutes, cooled and 2 ml of filter sterilised vitamin solution (page228) added. The media were dispensed into 100 ml sterile plastic jars in 20 ml aliquots, allowed to solidify and the jars weighed. Inocula of tissue grown on R.S. medium were added and the jars reweighed. The cultures were grown for 35 days under continuous light (Ediswan 'warm white' fluorescent 1500-1800 Lux) at $25^{\circ} \pm 1^{\circ}$. The tissues were removed from the jars, individually weighed and the growth indices calculated.

An analysis of variance was performed on the results and a histogram drawn of the mean growth index of the cultures grown on each medium.

(3) Variation of the volume of coconut water supplement in the induction medium

The nutrient stock solutions and sucrose for a 1 litre batch of M.S medium, excluding the coconut water supplement and vitamins, but including 50 ml of 0.2 mg/ml NAA in distilled water, were made up to 500 ml with distilled water. This nutrient concentrate was divided into 5 batches each of 100 ml and 10, 20, 30 and 40 ml of coconut water added to 4 batches. All five aliquots were made up to 200 ml with distilled water, the pH of each medium was adjusted to 6 with 1N potassium hydroxide, 1% w/v agar added and all the media were autoclaved for 15 minutes at 15 psi. Vitamin solution, 2 ml, was added, via a membrane filter, to each batch of cooled medium; 20 ml volumes were dispensed into 100 ml sterile plastic jars and allowed to solidify. Each jar of cold medium was weighed, inoculated with tissue grown on R.S. medium and reweighed immediately. The cultures were grown for 35 days in

continuous light (Ediswan 'warm white' fluorescent 1500-1800 Lux) at 25° ± 1°. The tissues were individually re-weighed and the growth indices calculated. An analysis of variance was performed on the results and a histogram drawn of the mean growth index for the tissues grown on each medium.

(4) The use of 2,4-Dichlorophenoxyacetic acid as a growth regulator

A solution of 200 mg/l of 2,4-D in distilled water was prepared (Solution A). Other solutions, containing 2,4-D 20 mg/l (Solution B) and 2 mg/l (Solution C), were prepared by dilution of Solutions A and B respectively. Five 100 ml batches of double strength MS medium nutrient solution, including a coconut water supplement (10% v/v at full dilution), were prepared as before. The following volumes of the 2,4-D solutions were each added to one batch of nutrient: 10 ml of Solution A, 10 ml of Solution B, 10 ml of Solution C and 1 ml of Solution C. To the other batch of nutrient was added 10 ml of a solution of NAA 200 mg/l. The nutrient concentrates were made up to 200 ml and treated as in previous experiments. The media were dispensed into sterile plastic jars in 20 ml aliquots and inoculated with known weights (approximately 50 mg) of tissue previously grown on R.S. medium. The cultures were grown for 35 days in continuous light at 25° ± 1°, reweighed, and the relative growth responses on the different media calculated as before.

(5) The growth response to NAA and kinetin

The method used for the preparation of the media was designed to keep the common constituents of the media uniform in each batch. The nutrient stock solutions (page228) and sucrose for a 4 l. batch of MS medium were made up to 2 l. with distilled water, mixed thoroughly and divided into 3 batches, each of 600 ml and 1 batch of 150 ml. Solutions containing 0.2, 0.02 and 0.002 mg/ml of NAA, in distilled water, were prepared and 60 ml of each added to one of the 600 ml trial batches of nutrient solution. To the small control batch of 150 ml was added 15 ml of the 0.2 mg/ml NAA solution. Each trial batch was again thoroughly mixed, made up to 800 ml with distilled water and sub-divided into 4 volumes of 200 ml. Solutions containing 0.3, 0.03 and 0.003 mg/ml of kinetin, in distilled water, were prepared and 10 ml of each added to one of the 200 ml volumes from each trial batch of nutrients. A 30 ml aliquot of coconut water was added to the 150 ml control batch of nutrient. Each volume of trial nutrient, and the control batch, was made up to 300 ml with distilled water in a 500 ml Erlenmeyer flask and the pH adjusted to 6 with 1N potassium hydroxide solution. After 1% w/v of agar had been added, each flask was sealed and autoclaved at 15 psi for 15 minutes. A vitamin solution was prepared (page228) and 3 ml added to each flask of cooling medium, after sterilisation by membrane filtration. The trial and control media were dispensed in 20 ml volumes into 100 ml sterile plastic jars and allowed to cool until the agar solidified. Each jar of medium was weighed, a piece of callus (50-100 mg) added and the jars immediately re-weighed. The callus used for the inoculation was taken from stock

cultures grown on RS medium. The fresh weights of the inocula were calculated and the cultures grown for 35 days at $25^{\circ} \pm 1^{\circ}$ in continuous light (Ediswan 'warm white' fluorescent tubes). After 35 days the tissues were removed from the jars and individually weighed. The growth indices were calculated and the results treated as before.

(6) Further investigation of the growth response to NAA and kinetin

A similar procedure was followed for the preparation of the media in the second experiment. A concentrated nutrient solution was prepared (2 l. for 4 l. of medium), divided (3 batches of 600 ml and one control batch of 150 ml) and the NAA solutions added as before. The trial batches were diluted (to 800 ml), sub-divided (4 x 200 ml) and kinetin added. Solutions containing 0.3, 0.15, 0.075 and 0.015 mg/ml of kinetin, in distilled water, were prepared and 20 ml of each added to one 200 ml volume of nutrient from each batch. Coconut water (30 ml) was added to the 150 ml control batch, as before. The trial and control batches were diluted to 300 ml, the pH of each medium was adjusted to 6, 1% w/v agar added, the media sterilised and sterile vitamin solution added. The media were dispensed into sterile plastic jars and callus tissue, grown on R.S. medium, added. The growth indices were calculated after a 35 day growth period.

(7) The growth response to 2,4-D and kinetin

The nutrient stock solutions and sucrose for a 5 l. batch of MS medium were made up to 2.5 l. with distilled water. Three trial batches of 750 ml and one control batch of 150 ml of the solution were taken. A series of solutions containing

0.15, .015 and .0015 mg/ml of 2,4-D in distilled water were prepared and 10 ml of each added to one trial batch of nutrient solution. A 15 ml aliquot of 0.2 mg/ml NAA solution was added to the 150 ml control batch. The total batches were made up to 1 litre with distilled water and sub-divided into volumes of 200 ml. Solutions containing 0.3, 0.03, 0.003 and 0.0003 mg/ml of kinetin in distilled water were prepared and 10 ml of each added to one 200 ml volume of each trial batch of nutrient. A 30 ml aliquot of coconut water was added to the control batch. The trial and control nutrient solutions were made up to 300 ml, treated as in the previous experiments and dispensed into 100 ml sterile jars (20 ml per jar). The trial media were inoculated with tissue grown on MS medium.(0.01 ppm 2,4-D 10% v/v coconut water) and the control with tissue grown on R.S. medium. The cultures were grown for 35 days in continuous light at $25^{\circ} \pm 1^{\circ}$ and the growth responses evaluated as before.

THE ADDITION OF MEVALONIC ACID TO THE CULTURE MEDIUM

A 10^{-1} molar solution was prepared by weighing 0.615 g of mevalonic acid lactone and making it up to 50 ml with a vitamin solution, containing 0.05 mg/ml of pyridoxine hydrochloride and nicotinic acid and 0.01 mg/ml of thiamine hydrochloride, in distilled water. Solutions containing 10^{-3}, 10^{-5}, 10^{-7} and 10^{-9} moles/litre were prepared by appropriate dilutions with vitamin solution.

Six 300 ml volumes of R.S. medium containing 1% w/v of agar were autoclaved at 15 psi for 15 minutes in sealed 500 ml Erlenmeyer flasks. To each flask of cooled, sterile, medium was added 3 ml of the appropriate MVA and vitamin solution sterilised by membrane filtration. The addition of 3 ml of 10^{-1} molar MVA solution to 300 ml of medium gave a 10^{-3} molar medium and 10^{-5}, 10^{-7}, 10^{-9} and 10^{-11} media were similarly prepared. A control medium containing no MVA was prepared by adding 3 ml of filter-sterilised vitamin solution. The same membrane filter was used for all the stock solutions and these were added in increasing order of strength. The media were mixed by swirling the flasks and poured in 20 ml volumes into 100 ml sterile plastic jars. One weighed piece of tissue (approximately 50 mg each), grown for 20 days on MS medium (10 ppm NAA, 10% v/v coconut water), was subcultured into each jar and grown at 25°C ± 1°C under fluorescent warm white light (1500-1800 lux) for 35 days. The tissues were individually weighed and the growth indices calculated as before.

PART III CHAPTER III

THE IDENTIFICATION OF PHYTOSTEROLS AND SAPOGENINS IN ONE YEAR OLD TISSUE CULTURES

THE EXTRACTION AND EXAMINATION BY T.L.C. OF PHYTOSTEROL AND SAPOGENIN FRACTIONS

A 5 g oven dried sample of tissue culture callus, from cultures which had been maintained for one year with, sub-culturing at 30 day intervals, was refluxed with 100 ml of 2N hydrochloric acid for 2 hours. The acid insoluble residue was filtered, (Whatman No.1), made alkaline with 10% ammonia solution, and dried overnight at 60° in a fan oven. The residue formed a solid lump which was powdered and extracted, with the filter paper, with light petroleum (B.P. 40-60°) in a Soxhlet apparatus for 24 hours. The solvent was removed on a rotary vacuum evaporator (Buchi) leaving an oily residue. A crude extract was obtained from a 2.5 g sample of whole Moroccan seed (RH.2336) by the same method. The dried residue obtained after acid hydrolysis did not require powdering prior to extraction.

The petrol-soluble crude extract from the callus was dissolved in 5 ml of chloroform and the extract from the seed in 10 ml of chloroform. A silica gel G T.L.C. plate (layer-thickness 250 μ) was activated at 110°C for one hour, cooled, and approximately 10 μl of each crude extract applied with a microcap. Standard solutions of diosgenin and sitosterol (1 mg/ml) were prepared and 10 μl of each applied

to the plate. Hexane:ethyl acetate 4:1 was used to run the plate in a Desaga 'S' chamber to a height of 15 cm. The solvent was allowed to evaporate from the plate, which was then sprayed with antimony trichloride 30% w/v in concentrated hydrochloric acid, and heated at 100°C for five minutes to develop the coloured spots.

Isolation of sapogenin and phytosterol fractions by preparative thin layer chromatography

The crude petrol-soluble callus extract was evaporated to dryness and redissolved in 2 ml of chloroform. The sample was applied as a band to eight, 250 μ, activated silica gel G T.L.C. plates with a melting point tube. The plates were run to a height of 15 cm using hexane:ethyl acetate 4:1 in an 'S' chamber. Each plate was covered with a sheet of glass, leaving a 1 inch margin at the edge exposed for spraying with the antimony trichloride reagent. The sprayed plates were heated to reveal the position of the phytosterol and sapogenin bands. The two bands were scraped from each plate and the bulked silica gel of each band, from the eight plates, placed in two Soxhlet thimbles which had been defatted by immersion in diethyl ether. The steroids were eluted from the silica gel with chloroform in Soxhlet apparatuses for six hours. The solvent was removed on a rotary vacuum evaporator and the extracts re-dissolved in 2 ml of chloroform.

The phytosterol and sapogenin were each re-applied to fresh T.L.C. plates and the procedure repeated. The final residues obtained were weighed, dissolved in 2 ml of chloroform and stored in 2.5 ml screw-cap vials ready for G.L.C. analysis.

THE IDENTIFICATION OF PHYTOSTEROLS AND SAPOGENINS

(1) Analysis of phytosterols by G.L.C.

A Perkin-Elmer F11 (Mark I) dual column gas chromatograph was used, with a flame ionisation detector and a Leeds and Northrup 'Speedomax W', 1 mV recorder. Three sets of columns were used in an initial G.L.C. investigation of the fractions.

(i) A pair of 1 metre glass columns, bore 3 mm, packed with a stationary phase of OV101 5%, coated on chromosorb G high performance, 80-100 mesh support material (Perkin Elmer). The columns were packed and conditioned by Jefferies[2].

(ii) A pair of 2 metre glass columns, of 3 mm bore packed with a stationary phase of OV-17 3%, coated on chromosorb G 80-100 mesh. The glass columns were made at Bath University and, prior to packing, were washed with 0.5 N hydrochloric acid, distilled water, 0.5 N methanolic potassium hydroxide and methanol until neutral. The columns were packed, with a combination of reduced pressure and vibration, until no further settling of the powder occurred. Conditioning of the columns was carried out at 210° for 4 days, then at 230° and 250° for 1 day and 270° for 2 days. The columns were not connected to the detectors during conditioning and a low carrier gas flow rate was used.

(iii) A pair of 6 ft stainless steel columns, bore ¼ inch, prepacked, by Perkin Elmer Ltd., with 2½ SE30 coated on chromosorb G 80-100 mesh. Conditioning was carried out at 200° for 4 days, then 220° and 240° for

I day and 250° for two days, under the conditions previously described.

The working temperature used with the columns were 250° for OVIOI and SE30 and 270° for OV-I7. The injection block was maintained at a temperature of 30° higher than the column working temperature. Nitrogen (oxygen free) was used as the carrier gas because of its low levels of water (<I5 ppm compared with 200 ppm in ordinary grade) and its low oxygen content (IO ppm compared with 0.5%). A molecular sieve (B.D.H. type 5A aluminium calcium silicate pellets) was fitted into the nitrogen line as a further precaution against moisture. The flow rate of the carrier gas was measured by attaching a teflon capillary tube plus soap bubble flowmeter to the unlit detector. Measurements were made at the working temperature of the column because it had been found that flow-rate fell with the increase in temperature.[2]

The conditions chosen for the analysis of T.M.Si-ethers of the phytosterol fraction were: 6 ft. ($\frac{1}{4}$ inch bore) SE30 2½%; oven temperature 250° and injection block temperature 280°; carrier gas: nitrogen at ml/min.

(2) The determination of phytosterol retention times relative to cholestane

An internal standard of 20 mg. of cholestane (99% pure, Applied Science Labs.) was weighed on torsion balance and dissolved in 20ml. of chloroform. Approximately 200 ug of the sterol fraction, previously prepared, was transferred to a 2.5 ml vial and 0.I25 ml of the internal standard solution added with an all-glass Agla micrometer syringe (Burroughs

Wellcome), of tested accuracy, 2 capacity 0.5 ml. The sample was evaporated to dryness in vacuo at 60° and 0.2 ml of B.S.A. reagent, bis-(trimethylsilyl)-acetamide (Phase Separation) added. The vial was tightly sealed, left at 60° for 30 minutes, after which, 3 µl injections of the mixture analysed by G.L.C., under the conditions previously described. Aliquots of 0.2 ml from standard sitosterol, stigmasterol, campesterol and cholesterol solutions, 1.0 mg/ml, (Applied Science Laboratories) were similarly silated and analysed by G.L.C. The retention times of the standards relative to cholestane were compared with the retention times of the components from the phytosterol extract.

(3) The G.L.C. analysis of the sapogenin fraction

The sapogenin fraction corresponding in Rf value to diosgenin was also analysed by G.L.C. as a T.M.Si-ether. The sample gave one peak and the relative retention time corresponded to that of the T.M.Si-ether of a diosgenin/yamogenin 3:2 standard solution.

(4) The infrared analysis of the sapogenin fraction

A 10 g sample of dried tissue culture callus, grown on RS medium and 10% v/v, was hydrolysed with 100 ml of 2N hydrochloric acid and extracted by the method previously described. Activity II silica gel was prepared by adding 50 g of activity I silica gel for adsorption chromatography (Woelm) to 5 ml of distilled water. The lumps were removed by shaking and the silica gel was allowed to stand for 1 hour prior to use. A glass chromatography column (30 cm long, bore 1.5 cm) was packed to a length of 15 cm with the activity II silica gel mixed to a slurry with hexane:ethyl acetate 9:1. The

flow rate was adjusted to 2 ml/minute and the column eluted with the same solvent. Ten ml fractions were collected and examined by T.L.C. The phytosterol was removed in the 90-130 ml fractions and retained for further investigations. The sapogenin was recovered in the 150-210 ml fractions. Considerable amounts of green pigment were present in the crude extract. The use of the hexane:ethyl acetate 9:1 for the whole elution reduced the amount of pigment removed with the sapogenin fraction. The solvent was removed from the bulked sapogenin fractions on a rotary vacuum evaporator and the sapogenin left at 60° under vacuum, overnight, to remove any traces of solvent which would interfere with the infrared analysis.

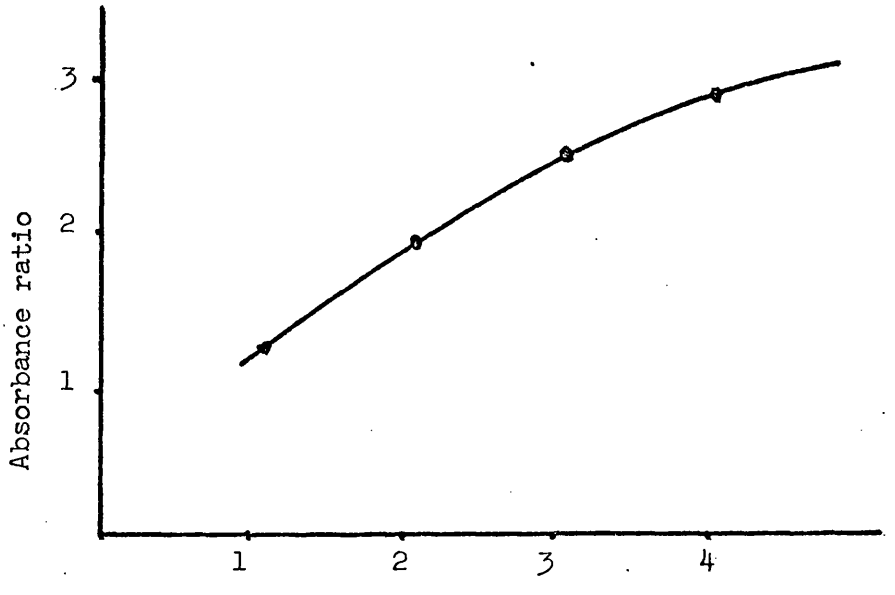
The analysis was carried out on a Hilger H800 double beam recording infrared spectrophotometer, with rock salt prisms, under the following conditions: 1 mm path length cell; slit 550 μ at 900 cm^{-1}; autoslit 25; gain 7; damping 4; scan speed 33 min/rev.

The sapogenin was dissolved in 0.5 ml of analar chloroform, transferred to the cell and the spectrum run twice over the 1050 to 850 cm^{-1} region.

(5) Diosgenin/yamogenin ratio of tissue culture and seed extracts

Jefferies obtained a calibration curve of the ratio of the absorbance values of the 900 cm^{-1} and 920 cm^{-1} peaks for a series of diosgenin and yamogenin standard mixtures, where diosgenin was the predominant compound (Fig.III.I). The absorbance values of these peaks for the sapogenin extracts of callus and seed were obtained and from the ratios of

Fig.III.1 Diosgenin:yamogenin ratio graph



Obtained from the absorbance ratio 900/920 against D:Y ratio and is used for mixtures containing more diosgenin than yamogenin. Reproduced from ref.2

$$\frac{\text{absorbance at 900 cm}^{-1}}{\text{absorbance at 920 cm}^{-1}}$$

the ratios of the diosgenin and yamogenin components were calculated.

(6) Mass spectrometric examination of the phytosterol fraction

The sterol, available from the column chromatographic separation of sapogenin, was bulked with sterol fractions obtained from other tissue extracts. The sterol extract (approx. 30 mg) was dissolved in a small amount of boiling methanol, (approx. 15 ml), under reflux, transferred to 5 ml centrifuge tubes and allowed to cool. The sterol crystals were collected by centrifugation, the methanol being removed with a pipette. The sterol was dried at 60°C and dissolved in 4 ml of chloroform, prior to further purification by preparative T.L.C.

Suitable T.L.C. plates were prepared by shaking 75 g of silica gel G, PF254-366, with 165 ml of distilled water. The slurry was spread over five plates (1 mm thick) and left overnight to air-dry, prior to activation at 110° for 1 hour. The sterol sample was applied as a band with a melting point tube and the plates run for 15 cm with hexane:ethyl acetate 3:1 in a chromatography tank. The sterol band (Rf 0.27) was detected by spraying the edge of the plate with the antimony trichloride reagent. The band was removed from each plate, the sterol eluted from the silica gel in a Soxhlet apparatus for 6 hours with chloroform and the composition of the extract determined by G.L.C. as a T.M.Si derivative.

Approximately 20 mg of the sterol fraction was recovered and half was transferred to a 25 ml round bottomed flask. One ml of pyridine and 2 ml of acetic anhydride were added and the mixture refluxed at 100°C for 1 hour. After cooling, 3 ml of methanol were added to the flask and solvent then removed under reduced pressure. The resulting acetates were dissolved in 1 ml chloroform. The procedure was repeated with 10 mg of sitosterol standard.

Silica gel G, 20% silver nitrate, plates were prepared and activated for 1 hour at 110°. The acetates were applied and the plate run with benzene:hexane 1:1 to a height of 15 cm in an 'S' chamber. The plate was sprayed with 50% sulphuric acid and heated at 110° for 10 minutes. The steryl acetate mixture gave one spot which corresponded with the sitosteryl acetate standard and had an Rf of 0.42.

The unacetylated sterol fraction was sent to the Tropical Products Institute, where a mass spectrometric analysis was carried out on the mixture and a G.L.C/mass spectrometric analysis of the major components performed.

PART III CHAPTER IV

THE DETERMINATION OF SAPOGENIN

THE COLOURIMETRIC ASSAY OF DIOSGENIN AND YAMOGENIN IN FENUGREEK TISSUE CULTURES

(1) Adsorption column chromatography of crude extracts

A 2.5 g Fenugreek seed sample was refluxed for two hours with 2N aqueous hydrochloric acid, the acid insoluble residue was filtered (Whatman No.1), made alkaline with 10% ammonia solution and dried overnight at 60° in a fan oven. The residue and filter paper were extracted with light petroleum (40-60° b.p.) for 24 hours in a Soxhlet apparatus and the solvent removed on a rotary vacuum evaporator. The petrol soluble extract was transferred to a 50 ml volumetric flask and made up to volume with chloroform.

Columns for adsorption chromatography were made from glass tubing of 0.5 cm. diameter / A 35 cm length of tube was heated at the end and drawn out to a fine tip. A glass wool plug and filter paper disc were inserted and pushed down to the base of the column. The outlet tip was closed with a piece of P.T.F.E. tubing, heat sealed at one end, and the column filled with hexane:ethyl acetate 9:1. The columns were packed with activity II silica gel. This was prepared by adding 10% v/w of distilled water to activity I silica gel for adsorption chromatography (Woelm), shaking to remove lumps and allowing it to stand for two hours prior to use. A slurry of the silica gel in hexane:ethyl acetate 9:1 was prepared and loaded into

the column with a fine tipped glass funnel. The P.T.F.E. closure was removed from the outlet tip to allow drainage of the displaced solvent. The column was vibrated to assist packing and was filled to a height of 24 cm. The flow rate was adjusted by filling the packed column with solvent and cutting off portions of the glass outlet tip until the aperture allowed the solvent to drain at a rate of 1-1.5 ml/minute.

A 1 ml sample of the diluted crude seed extract was evaporated to dryness, re-dissolved in hexane:ethyl acetate 9:1 (2 ml) and this solution applied to the column and eluted with the same solvent mixture. Fractions of 5 ml volume were collected, evaporated to dryness under vacuum at 60° and each re-dissolved in 0.5 ml of chloroform. A 10 µl sample of each fraction was applied to a silica gel G (250 µ) TLC plate which had been activated for one hour at 120°C. Standard solutions of sitosterol and diosgenin were prepared (1 mg/ml) and 10 µl of each also applied to the plate. Hexane:ethyl acetate 4:1 was used to run the plate to a height of 15 cm in a Desaga 'S' Chamber. The antimony trichloride reagent was used to spray the plate which was then heated at 100°C for five minutes to develop the spots. Fixed oil, sterol esters and dienes were removed from the column in the 0-10 ml fractions. Sterol was detected in the 10-40 ml fractions and sapogenin in the 35-55 ml fractions. The procedure was repeated with three more columns.

Four columns were run with 30 ml of hexane:ethyl acetate 9:1 and then with hexane:ethyl acetate 1:1. Fractions of 4 ml were collected after the solvent change. No sapogenin was detected after the third fraction (12 ml). A procedure

was adopted whereby 30 ml of 9:1 was used to remove the unwanted components, followed by 16 ml of 1:1 to recover the sapogenin fraction.

(2) Colourimetric assay

A 7 g sample of dry tissue was obtained by bulking together small samples of dried young callus. The sample was powdered, mixed and 1g, 2g and 4 g samples weighed. The samples were hydrolysed, dried, powdered and extracted as previously described. The partially purified sapogenin fractions were isolated, evaporated to dryness and made up to 5 ml in volumetric flasks. A 1 ml volume of each sample was transferred to a 5 ml centrifuge tube and evaporated to dryness under vacuum at 60°. Perchloric acid, 70%, 5 ml, was added to each tube with an Oxford variable volume dispenser. The mixture was agitated thoroughly for one minute with a Fisons Whirlimixer to produce vortex through the entire volume. After centrifugation at 3000 RPM for four minutes, in an MSE Minor centrifuge, the tube was removed and allowed to stand for a further five minutes. Approximately 3 ml of this solution was pipetted into a 1cm silica cuvette and the absorbance measured at 408 nm, against a reagent blank, using a Pye Unicam SP600 U.V. spectrophotometer, recording on a Smiths Servoscribe recorder. A fifth of the sapogenin extract from the 1 and 2 g samples gave a response of suitable magnitude for an assay. A callus sample of weight 1-2 g dry weight was chosen for the procedure.

(3) The calibration curve for the determination of diosgenin/yamogenin by the colourimetric method

A 20 mg sample of a standard diosgenin/yamogenin mixture 3:2 (95% pure), was made up to 100 ml in a volumetric flask with analar chloroform. Volumes of 1, 2, 3, 4 and 5 ml were transferred to a series of clean, dry 10 ml volumetric flasks with bulb pipettes and made up to volume. Volumes of 1 and 3 ml were similarly made up to 20 ml in volumetric flasks. A bulb pipette was used to transfer 1 ml quantities of each solution to clean, dry, centrifuge tubes (5 ml) to give weights of 10, 20, 30, 40, 60, 80 and 100 µg of standard. The absorbance value for each standard was determined, in a random order, by the colourimetric method. The procedure was repeated with four samples of each standard solution and the mean absorbance value obtained. A graph of absorbance against weight of diosgenin/yamogenin standard was plotted.

(4) Estimation of the error of the column recovery and colourimetric determination

A sample of sapogenin-free Fenugreek oil was prepared by extracting powdered Moroccan seed, RH.2336, with light petroleum in a Soxhlet apparatus for 24 hours. The yellow oil was refluxed with 2N hydrochloric acid for two hours. After cooling, the acidic aqueous phase was removed using a separating funnel and the oil washed with portions of distilled water, 10% ammonia solution and distilled water until the pH was neutral. Excess water was removed by pouring the oily mixture into a wet filter paper. The oil was dissolved in acetone and the acetone evaporated off under reduced pressure. The resultant clear yellow oil was examined by TLC and sterol, but no

sapogenin, was detected.

A 5 ml sample of a diosgenin/yamogenin standard solution previously prepared (2 mg/ml in chloroform) was pipetted into a clean, dry 100 ml volumetric flask. Approximately 100 mg of the sapogenin-free oil was weighed, dissolved in chloroform, transferred to the volumetric flask and the solution made up to volume with chloroform. A 5 ml aliquot of the solution was evaporated to dryness in a 100 ml round-bottomed flask.

An activity II silica gel column was prepared, as described previously, and the sample added to the column in 1 ml hexane:ethyl acetate 9:1 with a pipette. The flask was washed with four, 1 ml, volumes of the same solvent and the washings were added to the running column. The sapogenin fraction was collected in a 20 ml sample tube and evaporated to dryness under vacuum at 60°. The dry fraction was dissolved in chloroform and made up to 5 ml in a volumetric flask. Colourimetric determinations were carried out on two, (1 ml), samples of this solution. The procedure was repeated with nine columns. The volume of the 5 ml flask was checked, because the results obtained were higher than the theoretical value possible. The flask was washed with chloroform, dried and weighed. Analar chloroform was used to fill the flask to volume and it was re-weighed. The procedure was repeated twice and the mean weight of chloroform obtained. The true volume of the flask was calculated from the specific gravity of chloroform.

(5) Calibration of the volumetric flask

Wt. of chloroform in the flask: 7.2934

7.2796

7.2948

Mean wt. = 7.2892

Specific gravity of chloroform = 1.477

True flask volume = 4.94 ml

MODIFICATION OF THE PERCHLORIC ACID COLOURIMETRIC ASSAY

(1) Modification of the column separation procedure

A crude extract was obtained from a 1.5 g dried sample of young green callus. A glass chromatography column 45 cm in length, bore 0.5 cm, was made and packed to a height of 31 cm with activity II silica gel. The extract was applied to the column and eluted with hexane:ethyl acetate 9:1 as before. Fractions of 5 ml volume were collected and examined by TLC. Elution was continued with the same solvent until all the sterol and sapogenin had been removed from the column. The sterol was eluted in 40 ml and the sapogenin was present in the fractions 35-60 ml. The procedure was repeated three times and in two columns the separation of sapogenin and sterol was complete with sapogenin present in only the 40-60 ml fractions. The procedure was adopted and the 35-60 ml fractions were collected for the sapogenin assay.

(2) Estimation of the error for the modified colourimetry

A bulked sample of dried callus tissue (10 g) was powdered (Janke and Kunkel electric mill), sieved (mesh 1680 μ) and

Mixed, to obtain a uniform sample. Nine samples were weighed and the diosgenin/yamogenin content determined by the colourimetric method with the modified column separation procedure.

THE DETERMINATION OF DIOSGENIN/YAMOGENIN IN COTYLEDONS DURING THE INDUCTION OF TISSUE CULTURES

A sample of 400 selected Ethiopian seeds, RH.2602, was soaked in a 1% v/v β-propiolactone solution for 30 minutes at 50°C. The seeds were washed twice with sterile distilled water under aseptic conditions and transferred to petri dishes containing sterile 1% w/v agar in distilled water (20 seeds/dish). The seeds were incubated at 25°C for 12 hours in the dark. The cotyledons were aseptically removed from the viable seeds, which had become swollen and soft from the uptake of water. One cotyledon from each seed was cultured on R.S. solid medium and the other cotyledon retained for colourimetric assay of the diosgenin content. Twenty cotyledons were placed on each petri dish of medium (20 ml) and half the cultures grown in continuous light and half in continuous darkness, at 25° ± 1°. The moisture content of the bulked sample of cotyledons retained was determined and the samples assayed for diosgenin/yamogenin by the colourimetric method. Cotyledons grown on the culture medium were dried and assayed for sapogenin after 3, 7 and 20 days growth. The cultures chosen for assay at 7 and 20 days were those exhibiting signs of callus formation inthe form of undifferentiated cell mass at the cut edge of the cotyledon.

Fungal infection was detected in the cultures after the 20 day sampling and the experiment was terminated.

THE DETERMINATION OF DIOSGENIN/YAMOGENIN IN YOUNG TISSUE CULTURES INDUCED FROM FENUGREEK COTYLEDONS

A sample of 300 selected Moroccan seeds, RH.2336, was surface sterilised as before and placed on petri dishes containing sterile 1% agar in distilled water (10 seeds/plate). The seeds were incubated for five days at 25°C in the dark. The radicals emerged from the viable seed after two days and the cotyledons after four days. Fungal infection was detected in two dishes after five days and these were discarded. The cotyledons were aseptically removed from the five day old seedlings and half the sample grown on R.S. medium as before. The remaining cotyledons were dried and assayed by the colourimetric method. The cultures were grown in continuous light or darkness and a sample consisting of cotyledons on which callus growth was apparent assayed after six days. Healthy callus samples were excised from the original cotyledons and subcultured onto fresh medium after 27 days. The cotyledons and callus harvested were assayed as one sample. Subsequent subcultures and assays were performed at 54, 85 and 106 days.

PART III CHAPTER V

THE G.L.C. ASSAY OF SAPOGENIN AND PHYTOSTEROL

THE ASSAY OF DIOSGENIN/YAMOGENIN

(1) Adsorption column chromatographic separation of the sapogenin

Five dried tissue culture callus samples (3 g approx.) were each refluxed with 50 ml of 2N aqueous hydrochloric acid for two hours. To prevent solidification of the dried residues, 1 g of hyflo-super cell (Hopkins and Williams) was added to the flasks prior to filtration. After filtration (Whatman No.1) and washing with 10% ammonia solution, the residues were oven dried at 60° overnight. The residues and filter papers were extracted with light petroleum (b.p. 40-60°) in Soxhlet apparatuses for 24 hours. After removal of the solvent, the extracts were dissolved in 1 ml of hexane:ethyl acetate 9:1.

Five 45 cm long chromatography columns (0.5 cm bore glass tube) were packed with a slurry of activity II silica gel (prepared as before) in hexane:ethyl acetate 9:1 to a length of 31 cm. The flow rate of each column was adjusted to 1-1.5 ml/min. and the crude extracts were each loaded onto a column in 1 ml of the same solvent mixture. The flasks were each washed with a further 2 ml of solvent and the washings added to the running columns. Each column was eluted with a further 57 ml of the same solvent and 5 ml fractions collected in sample tubes. The fractions were dried under vacuum at

60°, redissolved in 0.5 ml of chloroform and 10 µl samples of each applied to a series of activated(250 µ) silica gel G TLC plates. Standards of diosgenin and sitosterol were also applied and the plates run to a height of 15 cm with hexane: ethyl acetate 4:1 in an 'S' chamber. The spots were developed with the antimony trichloride spray reagent and showed complete separation of sterol and sapogenin in three columns and incomplete separation on two columns. Sterol was found in the fractions 10-40 ml and sapogenin was first detected in the 40-45 ml fraction, where separation was complete. The procedure was repeated and incomplete separation occurred in four columns. The 35-40 ml fraction was found to contain both sterol and diosgenin/yamogenin. The sterol/sapogenin separation was not satisfactory for the GLC assay procedure and activity II silica gel was replaced with activity I silica gel. With the same procedure and solvent, complete separation of sterol and sapogenin was achieved in four trial columns. The sterol was removed in the 25-75 ml fractions, either sterol, or sapogenin, in the 75-80 ml fraction and sapogenin in the 80-105 ml fractions. A series of six further trials were carried out in which the solvent was changed to hexane:ethyl acetate 5:2 after the first 80 ml of elution with 9:1. Complete separation was achieved on all the columns and 10 ml of 5:2 was sufficient to remove the sapogenin from the column.

(2) GLC conditions of the diosgenin/yamogenin assay

Three of the sapogenin extracts obtained from the trial columns were prepared as T.M.Si-ethers and examined by GLC for the presence of sterol. The SE30 2½% columns were used, with the conditions previously described, and no trace of the major

sterol component, sitosterol, was detected.

A bulked sample of several dried callus cultures was powdered and mixed. Samples weighing 1, 2, 3, 4 and 6 g were weighed, hydrolysed and extracted as before. The diosgenin/yamogenin fractions were separated by column chromatography, collected in 20 ml sample tubes, evaporated to dryness under vacuum at 60°, and quantitatively transferred to 2.5 ml screw cap vials in a total of 2 ml of chloroform. To each vial 0.06 ml of the cholestane standard previously prepared (page244) was added and T.M.Si-ethers were prepared. Injections of 3 µl were made singly from each sample and the relative sizes of the internal standard and sapogenin peaks compared. The amount of diosgenin/yamogenin extracted from 2 or 3 g callus samples was sufficient for an assay determination with the quantity of internal standard added.

(3) The GLC determination of diosgenin/yamogenin as a T.M.Si-ether

A 6.65 mg sample (torsion balance) of a standard diosgenin/yamogenin mixture (3:2 and 95% pure by IR analysis) was weighed, transferred to a volumetric flask (calibrated volume 4.94 ml) and made up to volume with chloroform.

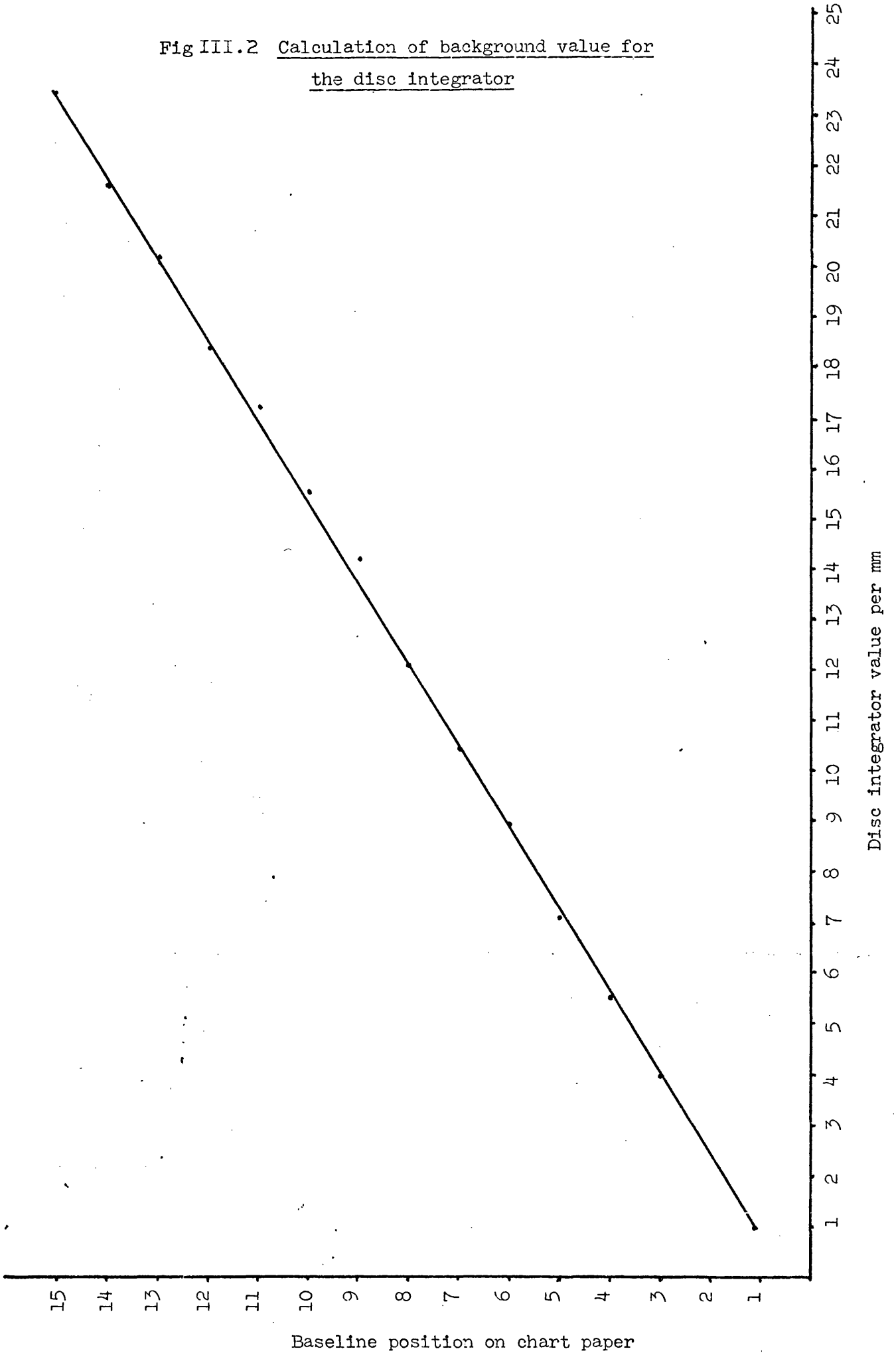
A series of 0.1, 0.2, 0.3, 0.4, 0.6 and 0.8 ml volumes were transferred to 2.5 ml screw-cap vials with the Agla micrometer syringe. To each sample was added 0.06 ml of the cholestane standard solution and T.M.Si-ether derivatives were prepared as before. Two injections from each sample were applied singly to the SE30 GLC column under the conditions previously described.

Quantitation of the peak sizes was by means of a Disc integrator fitted to the recorder. The integration zero was adjusted to coincide with the recorder zero and the integrator value per mm was obtained for each of the fifteen lines above the recorder zero.[2] The values obtained were used to construct a graph, Fig. III.2, which was used to calculate the background integrator value under each peak. This value was subtracted from the total integrator value of the peak, Fig. II.13 in the Results and Discussion shows a typical trace with disc integrator value measurement. The graphical method of estimating background count was found by Jefferies[2] to be easier than using the equation suggested by Disc Instruments.

(4) Estimation of the sapogenin recovery from the adsorption chromatography columns

Eight columns (45 cm on 0.5 cm) were packed with 31 cm of activity I silica gel. A standard solution containing 1 mg/ml of the sapogenin free Fenugreek oil (page 253) and 100 µg/ml of diosgenin/yamogenin standard was prepared and eight 2 ml aliquots transferred with a bulb pipette to round bottomed flasks. The chloroform was evaporated off on a rotary vacuum evaporator and samples quantitatively transferred to the columns in 1 ml of hexane:ethyl acetate 9:1. The flasks were washed with a further 4 ml of the same solvent and the washings added to the column. The diosgenin/yamogenin samples were collected and assayed by GLC as T.M.Si-ethers.

Fig III.2 Calculation of background value for the disc integrator



(5) The development of the hydrolysis and extraction procedure for tissue cultures

Five samples of approximately 2-3 g of dried callus tissue were hydrolysed as before, mixed with hyflo supercel, filtered, made alkaline, and dried overnight at 60°. Extraction of the residues and filter papers was carried out in Soxhlet apparatuses for 24 hours with light petroleum. The residues were re-extracted for a further 24 hours with analar chloroform and the second extracts evaporated to dryness. About 0.5 ml of chloroform was added to each residue and 15 µl applied to a TLC plate and co-chromatographed with standards of diosgenin and sitosterol. Three extracts showed traces of sterol and sapogenin.

The procedure was repeated but the initial extraction was carried out with chloroform. The subsequent extraction with fresh chloroform revealed the presence of no further sterol or sapogenin.

A 20 g sample of dried callus was powdered in an electric mill (Janke and Kunkel), sieved (mesh size 1680 µ), and mixed, to obtain a uniform sample. Ten samples of approximately 2.0g were weighed accurately and two samples were refluxed for times of ½, 1, 1½, 2 and 4 hours with 2N hydrochloric acid, (50 ml). The diosgenin/yamogenin fractions were extracted and assayed. A second series was hydrolysed for times of 1, 1½, 2, 3, 4 and 6 hours.

A time of 2 hours was chosen for the hydrolysis of tissue culture material with 2N hydrochloric acid.

(6) The determination of error for the extraction and GLC assay procedure

A uniform, powdered sample of callus was prepared and ten, 2.5 g, aliquots weighed accurately. The samples were hydrolysed, extracted, the sapogenin isolated, and assayed as described. The % range of error for the mean of two determinations was calculated at P = 0.05.

THE ASSAY OF THE FREE PHYTOSTEROL

(1) Extraction of the lipid fraction

Five samples of dried, powdered callus were extracted with light petroleum in a Soxhlet apparatus for 24 hours. The tissue was re-extracted for a further 24 hours with fresh petroleum. The solvent was removed from each extract on a rotary vacuum evaporator and each extract was dissolved in 0.5 ml chloroform. TLC analysis of the fractions showed the presence of sterol, but absence of sapogenin, in the first extract. The second extract contained neither sapogenin nor sterol.

(2) Isolation of the free sterol

In the absence of sapogenin in the crude extract, an activity II silica gel column, described on page 250 , was found to be satisfactory for the isolation of the sterol. The fixed oil and esters were removed with 10 ml of solvent (hexane: ethyl acetate 9:1) and the sterol fraction collected in the subsequent 35 ml.

(3) The GLC determination of sitosterol as a T.M.Si-ether

A standard solution of sitosterol was prepared (1.025 mg/ml) and 0.1, 0.4, 0.5, 0.6 and 0.8 ml samples transferred to 2.5 ml screw-cap vials with the Agla micrometer syringe. To each vial was added 0.06 ml of the cholestane standard, the solvent evaporated off, and T.M.Si-ethers were prepared. Two injections of each standard were singly injected onto the SE30 column and the peak areas calculated by the disc integrator and peak measurement method. In the latter method the height of the peak was multiplied by the peak width, at half the peak height. A linear calibration was obtained by both methods.

(4) Estimation of the sterol recovery from the adsorption chromatography column

Eight columns (45 x 0.5 cm) were packed with activity II silica gel in slurry with hexane:ethyl acetate 9:1. A standard solution of sitosterol containing 500 µg/ml (5 mg weighed on the torsion balance in a calibrated 10 ml volumetric flask) was prepared in hexane:ethyl acetate 9:1 and 0.5 ml applied to each of the columns with the Agla micrometer syringe. The columns were run, as before, and the sterol fraction recovered assayed as T.M.Si-ether derivatives. Two determinations were carried out on each sample and the % recovery calculated.

(5) The determination of the error of the sitosterol assay procedure

A 20 g bulked dried callus sample was powdered, sieved and mixed. Eight aliquots of approximately 1.5 g were weighed and extracted with light petroleum for 24 hours in Soxhlet apparatuses. The sterol fractions were isolated by column

chromatography and the sitosterol component assayed, the peak areas being calculated by the disc integrator method. The % range of error for the mean of any two determinations was calculated.

(6) Determination of the relative weight response of the sterols identified in culture extracts

A volumetric flask (2 ml) was calibrated, with chloroform, by weight. The true volume was found to be 1.92 ml. Standard solutions were prepared of cholesterol (1.22 mg/ml), campesterol (1.29 mg/ml), stigmasterol (1.04 mg/ml), sitosterol (1.19 mg/ml) and cholestane (1.03 mg/ml). A 0.12 ml sample of cholestane was placed in each of four 2.5 ml vials and with the Agla syringe and to each of the vials was added 0.5 ml of one of the other standards. T.M.Si-ether derivatives were prepared of each sample and the peak areas relative to cholestane determined by the Disc integrator measurement method. Two determinations were carried out on each sample and the mean ratio of sterol to internal standard peak area determined. The relative sensitivity was calculated by the formula

$$\frac{\text{Sterol peak area}}{\text{Cholestane peak area}} \times \frac{\text{wt.of cholestane}}{\text{wt.of sterol}}$$

PART III CHAPTER VI

A COMPARISON OF THE SAPOGENIN AND PHYTOSTEROL CONTENT OF CALLUS TISSUES GROWN ON NAA AND 2,4-D IN CONTINUOUS LIGHT

EXAMINATION OF THE FREE STEROL AND SAPOGENIN

Tissue from stock callus cultures which had been grown for six months on R.S. medium was transferred onto MS medium in which the NAA had been replaced by 0.01, 0.1 or 1.0 ppm 2,4-D. These cultures and the original stock culture were grown under identical conditions for a further six months. The basal medium for all four cultures was prepared in one volume and mixed thoroughly before the different growth regulator regimes were added. Subculturing was carried out at 30-35 day intervals and the cultures were grown in 100 ml sterile plastic jars containing 20-30 ml of medium. The temperature of the incubator room was maintained at $25^{\circ} \pm 1^{\circ}$ and continuous light was provided by fluorescent lights (Ediswan 'warm white') at 1500-1800 lux. After one year's growth the petrol soluble crude extract of hydrolysed tissue samples were examined for the presence of sterol and sapogenin by TLC.

Each culture was subcultured onto ten, 100 ml, batches of the appropriate medium in 250 ml Erlenmeyer flasks (300 mg of tissue per flask). The cultures were grown for 56 days and harvested. Sufficient tissue was retained for subculturing and the harvested callus was dried for 48 hours in a fan oven at 60°. Approximately 10 g of dry tissue was obtained from each set of flasks and each sample was powdered and mixed, to

provide a uniform batch. Two samples of about 2.5 g from each culture were hydrolysed, extracted and assayed for diosgenin/ yamogenin. Two 1.5 g samples were extracted with light petroleum for 24 hours and the free sterol fraction assayed.

A new culture was initiated on MS medium (1 ppm NAA, 10% v/v coconut water, pH 6.0) from the culture grown on R.S. medium. The five cultures were grown for a further 60 days in plastic jars with subculturing after 30 days. Another set of media was prepared in Erlenmeyer flasks and tissues again grown for 56 days. The free sterol and sapogenin extracted were examined as before.

EXAMINATION OF THE BOUND STEROLS

(1) Examination of the glycoside sterol fractions

Dry callus samples (1.5 g) were extracted for 24 hours with light petroleum, dried overnight at 60° to remove all traces of the solvent and refluxed with 2N hydrochloric acid for two hours. The residues were filtered, made alkaline and dried as before. The residues and filter papers were extracted with chloroform in Soxhlet apparatuses and the solvent evaporated off under vacuum. The diene, sterol glycoside and sapogenin fractions were separated by column chromatography using the method described on pageI33 . The sterol fractions were evaporated to dryness and each transferred to 2.5 ml screw cap vial in 2 ml of chloroform. Cholestane internal standard solution was added (0.06 ml) and the samples evaporated to dryness. A 0.125 ml volume of BSA reagent was added to each vial and T.M.Si-ethers formed. The samples were analysed by GLC on the 6 ft; SE30, columns as before.

(2) Examination of the sterol esters

The lipid fractions (fixed oil, sterol esters and free sterol) extracted with light petroleum, during the isolation of glycoside sterol fractions, were evaporated to dryness on the rotary vacuum evaporator. Activity II silica gel columns (31 cm x 0.5 cm) were prepared, as before, and each lipid fraction applied to a column in hexane:ethyl acetate 9:1 (2 ml). The ester and fixed oil fraction was eluted from the column with a further 9 ml of the same solvent mixture.

The oil and sterol ester fractions were evaporated to dryness in 50 ml round-bottomed flasks and approximately 20 ml of 0.5N alcoholic potassium hydroxide solution added to each flask. The samples were refluxed for one hour at 100° with frequent swirling of the flasks. The contents of each flask was washed into a separator with 30 ml of distilled water and, when cool, shaken with 3 volumes (20 ml) of diethyl ether. The ethereal solution was washed with 3 volumes (20 ml) of distilled water and transferred to a 100 ml round-bottomed flask. The ether was removed on a rotary vacuum evaporator and each residue dissolved in 50 ml of acetone, which was also removed by evaporation. The procedure was repeated with successive 20 ml volumes of acetone until the residue was dry. The dry residues were dissolved in hexane:ethyl acetate 9:1 and each applied to an activity II silica gel chromatography column (31 x 0.5 cm) as before. The column was eluted with a further 38 ml of the same solvent mixture and the fraction 10-40 ml collected and evaporated to dryness. Each residue was transferred to a 2.5 ml screw-cap vial in 2 ml of chloroform and T.M.Si derivatives prepared. The silated derivatives were analysed by GLC.

PART II CHAPTER VII

ALTERATION OF THE CONDITIONS EMPLOYED FOR TISSUE CULTURE GROWTH

THE EFFECT OF DIFFERENT LIGHT CONDITIONS ON THE DIOSGENIN/YAMOGENIN YIELD OF FENUGREEK TISSUE CULTURES

Callus cultures were maintained on RS medium for a period of I2 months, with subculturing at 30 day intervals. The cultures were grown either in continuous light (warm white fluorescent), or in continuous darkness. Two sets of ten Erlenmeyer flasks, each containing I00 mls. of RS medium, were inoculated with tissue from the two sets of cultures. The calluses were grown for a period of 56 days, the light-grown tissue in a continuous light of I500-I800 lux intensity. After the growth period, the cultures were subcultured and the harvested callus dried at 60°; powdered and assayed for diosgenin/yamogenin. The light conditions described were maintained for two further 56 day passages and the sapogenin yield determined.

After three passages, the light-grown callus was subjected to cyclic light and dark periods consisting of an I8 hour continuous photoperiod (I500-I800 lux) followed by a 6 hour dark period. The diosgenin/yamogenin yield of callus grown under these conditions was assayed and compared with that of tissue grown in total darkness. Cultures were grown for two 56 day passages.

THE GROWTH OF FENUGREEK TISSUE CULTURES ON MS MEDIUM CONTAINING 4-HYDROXYISOLEUCINE

A sample of 4-hydroxyisoleucine was extracted from Fenugreek seed and purified by Mr. I. M. Abul-Futuh. Three stock solutions each of 10 ml, containing 4-hydroxyisoleucine, 1, 0.1 and .01% w/v in vitamin solution, were prepared. A 2 l. batch of R.S. medium was dispensed into Erlenmeyer flasks (250 ml) in 100 aliquots. The flasks were sealed and autoclaved for 15 minutes at 15 psi after the addition of 1 g of agar (Oxoid No.1) to each. To five of the autoclaved, cooled, flasks 1 ml of vitamin solution, sterilised by membrane filtration, was added just before the agar set. The remaining 15 flasks were divided into three groups and similarly treated with 1 ml of the amino acid and vitamin stock solutions. The resulting media contained 0, 1, 10 and 100 ppm of 4-hydroxyisoleucine. The same membrane filter was used throughout and the amino acid solutions were added in increasing order of strength. The cold media were inoculated with tissue grown on R.S. medium (approximately 400 mg per flask). The cultures were grown for 18 hour photoperiods, 56 days under fluorescent warm white lights (Ediswan 1500-1800 lux) at $25^{\circ} \pm 1^{\circ}$, harvested, dried and assayed for diosgenin/yamogenin. The sterol recovered from the hydrolysed tissue was estimated.

A second experiment was carried out with concentrations of 1000, 100 and 0 ppm in the same medium. A 100 ml stock solution was prepared at each of the following concentrations of amino acid: 0.5%, .05% and .005% w/v. The 100 ml stock solutions were prepared containing 5 ml of

vitamin solution having 0.05 mg/ml of pyridoxine hydrochloride and nicotinic acid and 0.01 mg/ml thiamine hydrochloride. The inclusion of 20 ml of this dilution into 100 ml of MS medium provided the required vitamin content. Each solution was membrane sterilised with a Nalgene 0.2 μ disposable unit. A batch of MS medium containing the nutrients for 2 l. was prepared in 1600 ml and 80 ml dispensed into each of 20 Erlenmeyer flasks (250 ml). The flasks were autoclaved as before, after the addition of 1 g of agar to each. Twenty ml of sterilised amino acid solution was added to each flask of sterile medium with a sterile disposable syringe. A set of five flasks was prepared for each concentration of amino acid. A diluted vitamin solution, similarly treated, was added to five control flasks. The flasks were inoculated with tissue and grown as before.

A further 300 ml of each of the four media was prepared and aseptically transferred to sterile plastic jars in 20 ml aliquots before the agar was allowed to set. A known weight of tissue was grown in each pot for 35 days under the conditions described after which each culture was weighed and the growth indices calculated.

A COMPARISON OF THE EFFECT OF TWO VITAMIN FORMULATIONS ON THE GROWTH, SAPOGENIN AND STEROL YIELDS OF CULTURES

A 1.5 mg sample of cyanocobalamin was weighed (torsion balance) and made up to a solution of 100 ml with distilled water in a volumetric flask. A 1 ml aliquot was transferred to a fresh 100 ml volumetric flask with a bulb pipette. To the same flask was added folic acid 5 mg, riboflavin 5 mg, biotin, choline chloride, calcium pantothenate, pyridoxine phosphate and thiamine 10 mg of each, and nicotinamide 20 mg. The solution was made up to 100 ml with distilled water.

A 2 litre batch of MS medium at pH 6 containing 10 ppm NAA and 10 ppm kinetin, but containing no vitamins, was prepared. Volumes, each of 100 ml, were poured into 20 Erlenmeyer flasks (250 ml) and 1 g of oxoid agar No.1 added to each flask. The flasks were closed with non-absorbent cotton wool, covered with foil caps, autoclaved at 15 psi for 15 minutes and cooled. The vitamin solution was added to the cooling medium first before the agar set. Sterilisation of the vitamin solution was done by membrane filtration with a Sartorious filter An Everette luer lock 10 ml syringe was filled with the solution and the sterilised filter unit attached. A 1 ml aliquot was added to each of 10 flasks under aseptic conditions. A second 100 ml solution containing pyridoxine hydrochloride 5 mg, nicotinic acid 5 mg and thiamine hydrochloride 1 mg, was prepared. This solution was added in 1 ml volumes to each of the other 10 flasks using a fresh membrane filter unit and a clean syringe. To each flask two pieces of tissue (total weight approximately 200 mg),

grown on R.S. medium, were added. The cultures were grown in an incubator room at $25^{\circ} \pm 1^{\circ}$ under warm white fluorescent light 1500-1800 lux with 18 hours daily periods of light. After 56 days healthy green tissue was subcultured into fresh flasks of medium (ten for each medium) and the rest of the tissue harvested, dried for 48 hours at 60° and assayed for diosgenin/yamogenin.

A second subculture was carried out after a further 56 day culture period. During the third growth period the increase in fresh weight of cultures grown on the two media was also studied. To each of ten sterile plastic jars was added sterilised medium, (20 ml), containing the appropriate vitamins. Weighed pieces of tissue (50 mg) were added to each jar. These cultures were grown for 35 days under the same conditions, after which, the cultures were individually weighed and the growth indices calculated.

PART III CHAPTER VIII

INDUCTION AND MAINTENANCE OF SUSPENSION CULTURES

THE SELECTION OF A MEDIUM FOR SUSPENSION CULTURE

(1) The trial of MS medium with 10 ppm NAA and 10% v/v coconut water for the induction of suspension cultures

A 1 litre batch of R.S. liquid medium was prepared, and dispensed in 100 ml quantities into Erlenmeyer flasks (250 ml). The flasks were sealed with non-absorbent cotton wool plugs and foil caps and autoclaved for 15 minutes at 15 psi, in the absence of agar. Vitamin solution was prepared containing pyridoxine hydrochloride (0.05 mg/ml), nicotinic acid (0.5 mg/ml) and thiamine hydrochloride (0.01 mg/ml). One ml was added, to each flask of cooled medium, after sterilisation by membrane filtration. Healthy pieces of tissue (approximately 500 mg/flask) were transferred to the liquid medium from callus cultures, (grown on R.S. medium containing 1% agar), under aseptic conditions. The cultures were re-sealed and grown in a Gallenkamp orbital incubator at 25° under continuous fluorescent white light and shaken at 80 cycles/minute. The experiment finished when all the cultures died.

(2) The trial of a series of concentrations of NAA in MS medium

The nutrients for a 4 l. batch of MS medium, with a coconut water supplement equivalent to 10% v/v, at normal dilution, were made up to 2 l. with distilled water. The solution was mixed and divided into 4 batches each of 500 ml. A stock

solution of NAA (0.1 mg/ml) was prepared and volumes of 100, 10, 1 and 0.1 ml were each added to one of the batches of MS nutrient. The volume of each batch was made up to 1 litre, the pH adjusted to 6, the medium divided into 100 ml aliquots and dispensed into Erlenmeyer flasks, (250 ml). After autoclaving and the addition of vitamin solution, the media were inoculated with vigorously growing tissue (100 mg) from a 20 day old subculture, grown on R.S. medium. Each explant was transferred aseptically to a tared, sterile plastic jar and weighed before being added to the medium. The moisture content of two representative samples of the callus tissue used for explants was determined by drying the weighed samples at 60° for 48 hours and reweighing. The dry cell weights were calculated for the tissue explants added to the media from these moisture content determinations. The ten replicates for each medium were grown for 35 days, as before, and the experiment was stopped when cultures showed signs of dying, or had died. The initial tissue growth was bright green, but after 28 days some cultures had started to turn brown and after 35 days some were dead. The tissue was removed from the media by filtration with tared filter papers (Whatman No.1) and washed with distilled water. After drying for 48 hours at 60° the tissues and filter papers were weighed and the dry weight of the cultures calculated. The growth index for each culture was determined using the expression

$$\frac{\text{Final dry wt. of tissue - Dry wt. of initial explant}}{\text{Dry wt. of initial explant}}$$

(3) The trial of a series of coconut water concentrations with MS medium

Nutrients for a 4 l. batch of MS medium and sufficient NAA to give a concentration of 1 ppm at normal dilution, were made up to 2 l. with distilled water and thoroughly mixed. Four 500 ml aliquots were each mixed with one of the following volumes of coconut water: 0, 50, 100 and 200 ml. All four batches were made up to a volume of 1 litre with distilled water and the pH adjusted to 6. The media were divided into 100 ml quantities, autoclaved, vitamins added and inoculated as before. The cultures were grown for 32 days, examined and harvested. The dried tissues were weighed and the growth indices calculated.

(4) The trial of a series of kinetin concentrations

The nutrients for a 4 l. batch of MS medium and sufficient NAA to give a concentration of 0.1 ppm at normal dilution, were made up to 2 l. with distilled water. Three stock solutions containing 0.01, 0.1 and 1.0 mg/ml of kinetin, (6-furfuryl amino purine) were prepared and 1 ml of each added to one of four 500 ml aliquots of the nutrient solution. To the fourth aliquot was added 100 ml of coconut water. The volumes were each made up to 1 litre and the pH of the four media adjusted to 6. The media were dispensed in 100 ml volumes into Erlenmeyer flasks (250 ml) and treated as before. Known weights of tissue, from cultures on solid MS medium (1 ppm NAA and 10% v/v coconut water), were grown for 35 days and the increases in dry cell weight calculated.

(5) The trial of larger kinetin concentrations

The experimental procedure described in the previous experiment was repeated with four trial media containing 1, 2, 4 and 8 ppm of kinetin and a control containing 10% v/v coconut water. A series of 1 litre batches of each medium were prepared containing 0.1 ppm NAA and adjusted to pH 6. The increase in dry cell weight of tissue inocula, from callus cultures grown on MS solid medium (1 ppm NAA and 10% v/v coconut water), grown in these media, was recorded and the growth indices calculated.

(6) Alteration of the vitamin content of the suspension culture medium

A 2 l. batch of the suspension culture medium (MS medium with 0.1 ppm NAA and 2 ppm kinetin), containing no vitamins, was prepared. The medium was dispensed in 100 ml volumes into Erlenmeyer flasks (250 ml), autoclaved and cooled.

Kaul and Staba and Murashige and Skoog vitamin solutions, TableII [53], were prepared by the method previously described (page 226). One ml of the Kaul and Staba vitamin solution was added to each of ten flasks after filter sterilisation. Each of the other ten flasks was similarly treated with 1 ml of the Murashige and Skoog solution.

The flasks were all inoculated with tissue from the same callus culture and incubated, as before, in an orbital incubator.

THE MAINTENANCE OF AN ESTABLISHED SUSPENSION CULTURE

A 1 litre batch of MS liquid medium (0.1 ppm NAA, 2 ppm kinetin, pH 6) was prepared, dispensed in Erlenmeyer (250 ml) flasks in 100 ml volumes, autoclaved and vitamin solution added. Healthy tissue, from 25 day old subcultures of callus grown on MS solid medium (10 ppm NAA, 10% v/v coconut water) was used to inoculate the medium (approximately 200 mg/flask). Cultures were grown under the conditions previously described. After 30 days healthy growth had taken place in all flasks and tissue was subcultured into fresh flasks of medium, prepared as before.

Lengths of glass tubing 25 cm long, bore 0.5 cm, were plugged at one end with non-absorbent cotton wool. The tubes were wrapped in aluminium foil and dry heat sterilised at 150° for 5 hours. A rubber pipette bulb was attached to the plugged end of the tube, which was used as a wide aperture pipette to aseptically transfer small amounts of tissue and exhausted medium to the flasks of fresh medium. Tissue was taken from flasks containing the healthiest looking cultures for sub-culturing. Two flasks of the initial suspension culture were retained un-opened for a week after subcultures were performed, but the rest of the tissue was harvested, washed, and dried at 60° for 48 hours. No sign of contamination was detected in the subcultures after one week and the two flasks of culture retained were harvested. The subcultures were slow to establish themselves and 60 day growth was required before they were again ready for harvesting. A second subculture was carried out by the same procedure and these cultures were successfully growing at the time of writing (July 1974).

PART III CHAPTER IX

METHODS OF STATISTICAL ANALYSIS

(i) CONFIDENCE INTERVALS

Estimating a confidence interval is the process of inferring, from the properties of the sample, within what range a parameter probably lies.[3] Confidence intervals were calculated for the data in Tables II.5& II.7 using the expression

$$\mu = \bar{x} \pm t\,(S_{\bar{x}})$$

where $$S_{\bar{x}} = \sqrt{\frac{\Sigma x^2 - \frac{(\Sigma x)}{N}}{(N-1)\,N}}$$

t = t-distribution at $P = 0.05$

$\bar{x}$ = the sample mean

μ = the true population mean

N = the number replicates

(ii) ANALYSIS OF VARIANCE OF THE GROWTH RESPONSE OF TISSUE

Analysis of variance[3] was used to compare the increase in fresh weight of groups of callus replicates grown on different media. In TableIII.2 the F value determined by the calculation was 20.56. From Tables the critical F value at $P = 0.01$ (4-45) was 3.83. This means that the differences occurring between groups of results was greater than the differences occurring within groups. The minimum significant difference between the different groups at $P = 0.05$ was calculated using the Studentized Range Test. From TableIII.2 it can be seen that a difference in the mean growth indices of greater than 35.86 indicated a significant difference in growth response between two groups of replicates. The minimum significant difference has been expressed on the histograms of growth response in the results and discussion.

Table III.2
Analysis of Variance of NAA variation in induction medium trial

$(\Sigma x)^2 = 19495668.85$

$\Sigma x^2 = 492185.00$ $\quad Tm^2 = 4560228.20$

Preliminary calculation

	Total of squares	Number of items squared	Obs./squared item	Total squares/ observation
Grand	19495668.85	1	50	389913.44
Media	4560228.20	5	10	456022.82
Observ.	492185.00	50	1	492185.00

Analysis of Variance

	DF	SS	Variance estimate
Media	4	456022.82-389913.44 = 66109.38	16527.34
Error	45	492185.00-456022.82 = 36162.20	803.60
Total	49		

$$F = \frac{16527.34}{803.60} = 20.56$$

sig F at P = .01 is 3.83 (tables)

Studentized Range test minimum significant difference

$$K = 4.0 \times \sqrt{\frac{803.6}{10}} = 35.86$$

Table III.3

Analysis of Variance of coconut water variation trial

$\Sigma x = 965.6$ $(\Sigma x)^2 = 932518.54$

$\Sigma x^2 = 21926.81$ $Tm^2 = 202929.067$

Preliminary calculation

	Total of squares	Number of items squared	Obs./squared item	Total squares/ observation
Grand	932518.54	1	50	18650.37
Media	202929.07	5	10	20292.91
Observ.	21926.81	50	1	21926.81

Analysis of Variance

	DF	SS	Variance est.
Media	4	20292.91-18650.37 = 1642.52	410.63
Error	45	21926.81-20292.91 = 1633.9	36.31
Total	49		

$F = \frac{410.63}{36.31} = 11.30$ P = .01 F = 3.83 from tables

$\therefore$ significant

Studentized Range Test

$$K = \overset{*}{4.00} \times \sqrt{\frac{36.31}{10}} = 7.62$$

* from tables

TableIII.4

Analysis of Variance 2,4-D growth regulator trial

Analysis

Source	DF	SS	Variance estimate
Media	4	1344.9	336.2
Error	45	1818.0	40
Total	49		

F = 8.4 Sig. F at P = .01
3.83

Studentized Range Test value

P = .05 Sig. range 9.4

Table III.5

Analysis of Variance of the first trial of NAA and kinetin

Preliminary calculation

	Total of squares	No. of items squared	No. of obs./ squared item	Total squares/ observation
Grand	22526224.6	1	195	115519.1
Media	2550262.19	13	15	170017.5
Observ.	189005.7	195	1	189005.7

Analysis of Variance

Source	DF	SS	Variance Estimate
Media	12	54498.4	4541.5
Error	182	18988.2	104.3
Total	194		

F = 43.5 at P = .01 F = 2.3

Studentized Range Test

P = .05 sig. range 12.34

Table III.6

Analysis of Variance for the second trial of NAA and kinetin

Preliminary calculation

	Total of squares	Number of items squared	Obs./squared item	Total squares/ observation
Grand	20178243.68	1	195	103478.1
Media	1713689.41	13	15	114245.96
Observ.	126942.0	195	1	126942.0

Analysis of Variance

Source	DF	SS	Variance estimate
Media	12	10767.86	897.30
Error	182	12696.00	69.75
Total	194		

F = 12.89 at P = .01 F = 2.3

Studentized Range Test

P = .05 Sig. range = 10.1

Table III.7

Analysis of Variance for the trial of 2,4-D and kinetin

Preliminary calculation

	Total of squares	No. of items squared	No. of obs./ squared item	Total squares/ observation
Grand	74628692.68	1	240	310952.88
Media	5388331.81	16	15	359222.12
Observ.	422879.85	240	1	422879.55

Analysis of Variance

Source	DF	SS	Variance estimate
Media	15	48269.2	3217.94
Error	224	62657.73	284.18
Total	239		

F = 11.32

at P = .01 F = 2.10

Studentized Range Test

P = .05 sig. range = 20.43

(iii) CALCULATION OF ERROR FOR THE ASSAY PROCEDURE

The standard method for calculating standard deviation was used,[3] involving the calculation of Σx^2 and $(\Sigma x)^2$ values and substitution into the expression

$$S^2 = \frac{(\Sigma x^2) - \frac{(\Sigma x)^2}{N}}{N-1}$$

where S = the standard deviation

N = the number of samples

x = the experimental value for each sample

The error was expressed as a percentage of the mean, i.e. coefficient of variation

$$CV = \frac{S}{\bar{x}} \times 100$$

The % range of error at P = 0.05 was calculated from

$$\bar{x} \pm t \frac{CV}{\sqrt{N}}$$

where $\bar{x}$ = the experimental sample mean

t = t-distribution at P = 0.05

(iv) CALIBRATION CURVE REGRESSION ANALYSIS

The least squares straight line was calculated using linear regression analysis.[3]

The equation for the true regression line is

$$\tilde{y} = a + bx$$

where a = the intercept on the y axis at x = o (the regression constant)

b = the slope (the regression coefficient)

Since $a = \bar{y} - b\bar{x}$ where $\bar{x}$ and $\bar{y}$ are the means of x and y observations the least squares line will pass through the point $\bar{x}\bar{y}$.

The slope b is given by the expression

$$b = \frac{\Sigma xy - \frac{(\Sigma x)(\Sigma y)}{N}}{\Sigma x^2 - \frac{(\Sigma x)^2}{N}}$$

(v) ESTIMATION OF THE ERROR VARIANCE AND CONFIDENCE INTERVAL FOR THE REGRESSION LINE

The error variance is given by [3]

$$S_{y.x}^2 = \frac{1}{N-2}\left\{\Sigma y^2 - \frac{(\Sigma y)^2}{N} - b\left[\Sigma xy - \frac{(\Sigma x)(\Sigma y)}{N}\right]\right\}$$

The 95% confidence interval for $\tilde{y}$ at $\bar{x}$ is given by

$\tilde{y} = \bar{y} \pm t\,(S_{\bar{y}})$ where t has (N-2) degrees of freedom

$$S_{\bar{y}} = \sqrt{\frac{S_{y.x}}{N}}$$

The calculations of the linear regression analyses for the GLC calibrations of diosgenin/yamogenin and sitosterol are given in Tables III.8 & III.9

Table III.8

Calibration curve for diosgenin/yamogenin using cholestane as internal standard by GLC

X	Y	XY
135	1.48	199.8
270	3.30	891.0
405	4.81	1948.0
540	6.63	3580.2
810	10.22	8278.2
1080	13.54	14623.2

Σx 3240.0

Σy^2 367.9

Σx^2 2369250.0

Σy 39.98

$\Sigma xy = 29520.45$

$\bar{x} = 540$

$\bar{y} = 6.66$

$$\text{slope } b = \frac{\Sigma xy - \frac{(\Sigma x)(\Sigma y)}{N}}{x^2 - \frac{(\Sigma x)^2}{N}}$$

$$b = \frac{7931.25}{619650.0} = .0127$$

$$\text{Error variance } Syx^2 = \frac{1}{N-2}\left\{\Sigma y^2 - \frac{(\Sigma y)^2}{N} - b\left[\Sigma xy - \frac{(\Sigma x)(\Sigma y)}{N}\right]\right\}$$

$$Syx^2 = 0.1933$$

$$Sy^2 = \frac{Sy.x^2}{N} = .0322 \qquad Sy = 0.179$$

$$\tilde{y} = 6.66 \pm t(0.179) \qquad t \text{ at } 4DF \text{ and } P=.05 = 2.78$$

$$= 6.66 \pm .49$$

$$= 6.16 - 7.15$$

Table III.9

Calibration curve for sitosterol using cholestane as internal standard by GLC

Disc Integrator Method

$\Sigma x = 2461.44$ $\Sigma y = 26.85$

$\Sigma x^2 = 1493704.36$ $\Sigma y^2 = 181.39$

$(\Sigma x)^2 = 6058686.87$ $(\Sigma y)^2 = 720.92$

$xy = 16456.15$

Slope = 0.01148

Error variance $Sy.x^2 = 0.01092$ $Sy^2 = .002$ $S\bar{y} = .046$

Confidence interval $\tilde{y} = 5.37 \pm 0.146$

or $5.37 \pm 2.72\%$

Peak Measurement Method

$\Sigma x = 2461.44$ $\Sigma y = 28.88$

$\Sigma x^2 = 1493704.36$ $\Sigma y^2 = 206.36$

$(\Sigma x)^2 = 6058686.87$ $(\Sigma y)^2 = 834.05$

$\Sigma xy = 17556.633$ slope = 0.01184

Error variance $Sy.x^2 = .0111$ $Sy^2 = .002$ $Sy = .047$

Confidence interval $\tilde{y} = 5.78 \pm 0.149$

Table III.IO

Suspension culture

The growth response to a series of concentrations of NAA in MS medium

Analysis of Variance

Source	DF	SS	Variance estimate
Media	3	26978	8992.7
Error	36	20184	560.7
Total	39		

F = 16.04 At P = .01 F = 4.51

Studentized Range Test

P = .05 Range = 35.9

The growth response to a series of coconut water concentrations

Analysis of Variance

Source	DF	SS	Variance estimate
Media	3	224940	74980
Error	36	100921.8	2803
Total	39		

F = 26.75

Studentized Range Test

K = 80 P = .05

Table III.II

The growth response to a series of kinetin concentrations in the induction of suspension cultures

Analysis of Variance

Source	DF	SS	Variance Estimate
Media	3	281574.3	93858.1
Error	36	271813.4	7550.3
Total	39		

P = ·05 F = 12.43

Studentized Range Test K = 131

The growth response to a series of kinetin concentrations in the induction of suspension cultures

Analysis of Variance

Source	DF	SS	Variance Estimate
Media	4	19794.15	4948.53
Error	55	64012.39	1163.86
Total	59		

P = ·05 F = 4.2518

Studentized Range Test K = 50.00

Table III.I2

Growth response to 4-hydroxyisoleucine in the media at different concentrations

Analysis of Variance

	DF	SS	VE
Media	3	630	210
Error	56	8135.8	145.28
Total	59		

$$F = \frac{210}{145.3} = 1.44$$

F value at P = .05 = 2.84

∴ no significant difference between media

REFERENCES

PART 3 - EXPERIMENTAL

I. Street, H. E. Plant tissue and cell culture, I973, pp I-30
Ed. H. E. Street, Blackwell Press, Oxford.

2. Jefferies, T. M. Ph.D. Thesis, Bath University, I973.

3. Goldstein, A. Biostatistics, Macmillan, New York, I964.